PHYSIOLOGICAL ONTOGENY IN PLANTS AND ITS RELATION TO NUTRITION

1. THE EFFECT OF NITROGEN SUPPLY ON THE GROWTH OF THE PLANT AND ITS PARTS

by

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With a Statistical Appendix by

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INTRODUCTION.

The growth and development of an organism is the result of a large number of directed and integrated metabolic reactions. The integration is such that there are temporal drifts in metabolism which underlie drifts in size, structure, chemical composition, and in the rates of change of these attributes; these drifts can be recognized as characteristic features of ontogeny. Integration is not confined to direct relations among chemical or metabolic reactions, but also involves the changes in structure and composition, which themselves influence the reactions that caused them. The elucidation of this complex integration represents the ultimate task in the study of growth; it can be achieved only after intensive investigation of the nature of ontogenetic drifts of various kinds and of their interrelations. To this investigation the present work forms a contribution.

It is known that the chemical composition of the plant not only drifts with time, but varies with the nutrient supply. It has therefore been considered that the observation of ontogenetic drifts under different initial supplies of nutrients should be a valuable method of approach to the study of physiological ontogeny. This paper, apart from the presentation of certain introductory conceptions, concerns the definition of the effect of nitrogen supply on the drifting size and growth rate. The observations have been confined mainly to annual or annually renascent gramineous plants; in these the drifts with which we are concerned are shown in comparatively simple form.

[1] This represents one of a series of investigations carried out at the Waite Institute and financed co-operatively by the Carnegie Corporation of New York, the Council for Scientific and Industrial Research, and the University of Adelaide.

GROWTH STAGES.

The life-history of a single vegetative organ such as a leaf is marked by three phases: (*a*) an initial period of growth which we shall term adolescence; (*b*) a period of constant weight after the cessation of growth, known as maturity; and (*c*) a period of decline in weight and internal disorganization known as senescence. By analogy, these terms may be applied to the totality of one type of organ on the plant, such as the leaves. The terms then become only average expressions of the ontogenetic state of the organs; thus, when the leaves as a whole cease to increase in weight, some will be declining in weight and others increasing. The analogy, however, is justified since the general ontogeny of the totality of organs of one type reflects that of a single organ of the same type.

RELATIVE GROWTH RATE AND ITS COMPONENTS.

The primary measure of the rate of change in size that has been adopted is the relative growth rate, R, given by the equation

$$R = \frac{1}{W}\frac{dW}{dt}, \tag{1}$$

where W is the dry weight of the whole plant and t the time. The characteristic time curve of this rate has been demonstrated by Briggs, Kidd, and West (1920). The relative growth rate is a convenient measure for comparing rate of increase in dry weight at different times and in different plants, but on account of the heterogeneity of the plant it has no direct physiological significance. It has been resolved, however, into two components, the fraction of the plant that is producing new material and the rate of production by this fraction.

The new material is produced mainly by the leaves, and the fraction of the plant that they represent can be measured as leaf weight ratio, F_W, given by the equation

$$F_W = W_L/W, \tag{2}$$

where W_L is the total leaf weight. It can also be measured as leaf area ratio (F_A), substituting the total leaf area (A_L) for W_L. The time curve of F_A has been described by Briggs, Kidd, and West (1920) and by Inamdar, Singh, and Pande (1925). The latter authors showed that under conditions of bright sunlight, W_L/A_L tends to be constant; on the other hand, a complex drift of this ratio was recorded for single successive mature leaves of barley by Gregory and Richards (1929); Smirnow (1928) found in *Helianthus* and *Nicotiana* that the average value of the ratio for all but the lower leaves increased with time during the growing period; and for the total leaves of *Fagopyron* Maiwald (1930) found it to fall. In the present work we have measured leaf weight, since the measurement of leaf area was impracticable.

The second component of the relative growth rate is the increase in dry weight

of the plant per unit of leaf, the unit leaf rate (E) of Briggs, Kidd, and West, which is given by the equation

$$E_A = \frac{1}{A_L}\frac{dW}{dt}. \tag{3}$$

We have used the analogous quantity

$$E_W = \frac{1}{W_L}\frac{dW}{dt}. \tag{4}$$

Since by definition $R = FE$, we may expect from the observations of Inamdar, Singh, and Pande that E will rise and then fall, although this expectation might apply only to their environmental conditions. In any case, since the assimilatory activity of the seedling leaves of those plants in which these do not act as storage organs increases at first, an initial rise in E would in such plants be expected, as Briggs, Kidd, and West point out. A subsequent fall would be caused by a decrease in assimilatory activity of the individual leaves as they grow older, and the increasing mean age of the leaves as a whole. Evidence[2] for such a decrease has been obtained by Henckel and Litvinov (1930) and by Gassner and Goeze (1934*b*). Respiration also, as Briggs, Kidd, and West point out, will make the unit leaf rate curve still more concave to the time axis.

Briggs, Kidd, and West calculated values for E_A from some data for maize, and demonstrated an initial rise. During the middle portion of the growing period, the values showed marked fluctuations about a mean, these being attributable mainly to temperature. The subsequent values they regarded as inaccurate. Wagner (1932) gives a set of data for oats from which E_W can be calculated over the later part of the life-period; a general decline with time is shown. There is a similar decline in E_A (up to leaf maturity) in some experiments of Gregory (1926). Both Wagner and Gregory, however, were concerned with plants senescing under conditions of falling temperature, and Gregory attributes the fall in unit leaf rate in his case solely to falling temperature[3].

It has thus been shown that relative growth rate can be represented as the product of two factors: the one is the net rate of increase of dry matter resulting from a number of metabolic processes, the rates of all of which except carbon assimilation are generally relatively small; the other is an easily measurable aspect of morphological structure. Both factors exhibit characteristic ontogenetic drifts. In the present paper these conceptions will be used in describing the effect of nitrogen supply on growth. The description will be based largely on two experiments performed in this laboratory, the details of which will be given in the following section.

[2] Singh and Lal (1935) have studied age-assimilation relations, but they do not give data for the drift in assimilation rate of all the leaves or a single leaf throughout the life-period under normal environmental conditions.

[3] Evidence will be given subsequently that E_W falls independently of temperature.

PRESENTATION OF NEW DATA.

Experiment 1.

On June 23, 1933, seeds of a pure line of late Gluyas wheat (*Triticum vulgare* Vill.) were planted in glazed earthenware pots containing 25 kg. water-washed sand. Fifteen seeds were planted in each of 120 pots, and about 25 seeds in each of a further 12 pots. On July 4, before the second leaf had appeared, the seedlings of the 120 pots were thinned to 6 uniform plants per pot, and those in the remaining pots were thinned to a similar standard of uniformity. At the same time, nutrient solutions were added so that each pot received the following amounts:

KH_2PO_4	0·80 gm.
K_2SO_4	2·00 ,,
$MgSO_4.7H_2O$	0·50 ,,
$CaCl_2.6H_2O$	2·00 ,,
$FeCl_3$	0·08 ,,
$MnSO_4.4H_2O$	0·0021 ,,

The pots were divided into 4 groups each receiving a different amount of $NaNO_3$. The pots of these groups will be referred to respectively as those of treatments I–IV. The amounts of $NaNO_3$ were as follows:

treatment I	1 gm.
II	3 ,,
III	6 ,,
IV	10 ,,

Distilled water was added frequently in such amount as to restore the moisture content of the sand to 50 per cent. of its maximum water-holding capacity. Evaporation loss was restricted by spreading 2·75 kg. of fine gravel on the surface of each pot, and the depletion of water never exceeded half the amount originally present.

Harvests were made on July 4, 10, 13, 18, and 31, August 28, September 19, October 13, 31, and November 9. The first harvest consisted of 5 replicates, each consisting of the shoots of 24 plants similar to those left in the thinning operation. The second harvest was made from the closely planted pots, and consisted of 5 replicates of 5 plants each per treatment. The third and fourth harvests were made by thinning of the 6 plants in each pot to 4 evenly spaced ones. The 5 replicates of treatments I, II, and III each contained 5 plants, and treatment IV had 3 replicates of 4 plants each; some of the pots of treatment IV had been rejected owing to the plants being injured at the time of adding the solutions. Harvests 5–10 consisted of the 4 remaining plants of 5 pots for each treatment except treatment IV, for which at harvest 5 no pots, and at harvest 6 only 3 pots, were available.

Previous experiments had led to the conclusion that, at the time of harvesting from pots with more than 4 plants each the fraction of nutrients removed from the sand was negligible, so that no complication was anticipated from the procedure adopted, which was necessitated by the number of pots available.

Various portions of the plants were separated at harvesting. First leaves were separated for harvests 1–4, second leaves from their appearance till harvest 5, and third leaves till harvest 7. Leaf separations were made by cutting at the ligule but, in the case of the youngest leaf of each axis, only that portion was taken which had protruded from the sheath of its predecessor. Tillers appeared at harvest 5, and from this harvest onwards the primary shoot was kept separate. The stem fraction included true stems, the leaf sheaths, portions of immature leaves and also, although these would be of negligible weight, coleoptiles and rudimentary tillers. Inflorescences, which first appeared on September 27, were separated at harvests 8–10 by cutting immediately beneath the lowest floret. From harvest 6 onwards, roots were obtained by sieving under water until practically free from sand.

Immediately after separation, the various portions of the plants were weighed, and dried at 100°C. for one night, and then at 80°C. to constant weight. The material was then ground for analytical purposes. Estimations of sand content were made on the root material, and the dry weight data corrected for the small quantities present.

In all cases the results have been expressed on a basis of one plant per replicate (see Table 2).

TABLE 1.

Harvest Details of Experiment 2.

Harvest.	Date.	No. of plants per pot.	No. of replicates.
2	Feb. 6	12	6
3	„ 18	7	6
4	„ 25	4	5
5	Mar. 12	3	5
6	April 2	3	5
7	„ 29	3	5

The date of appearance of the first inflorescence and of the exsertion of the first anthers was noted for each plant. From these was obtained for both phenomena for each treatment the date for the $\frac{1}{2}n$th plant, n being the number of plants still unharvested at that date. These dates are indicated in the graphs.

Tiller counts were made on each plant at harvesting from harvest 6 onwards, and the average number of tillers per plant recorded (see Table 6).

Experiment 2.

On January 16, 1935, seeds of Sudan grass (*Andropogon sudanensis* Leppan and Bosman) were planted in glazed earthenware pots containing 14 kg. water-washed sand, and also in seed-pans containing similar sand. Harvest 1 was taken on January 25, and comprised 15 seed-pans each containing 48 seedlings. Six of these sets of 48 were taken as replicates for dry weight determination. The remaining harvests were made from the pots, all of which were thinned to their final number of plants on January 25. On January 27, nutrient solutions were added so that each pot received the following amounts:

KH_2PO_4	0·60 gm.
K_2SO_4	2·00 „
$MgSO_4.7H_2O$	0·40 „
$CaCl_2$	0·74 „
$FeCl_3$	0·06 „
$MnSO_4.4H_2O$	0·0016 „

The pots were divided into three groups each receiving a different amount of $NaNO_3$ per pot. The amounts will be referred to as treatments I–III; they were as follows:

I	0·75 gm.
II	2·25 „
III	4·50 „

This was almost identical with the treatment used in a previous experiment with the same species (Ballard 1933). The moisture content of the sand was maintained at 57 per cent. of its maximum water-holding capacity. Harvest details are given in Table 1.

The plant portions were weighed and dried to constant weight at 85°C. under forced draught. The root weights were again corrected for sand content. In all cases the results are expressed on a basis of one plant per replicate (see Table 3).

At each harvest measurements were made of the respiration rate of leaves from pots not used for dry weight determinations.

The inflorescences and flowering data were obtained as in Experiment 1, except that all the plants in a pot instead of the single plant were takes as the unit.

Calculation of Derived Quantities.

Relative growth rates, leaf weight ratios, and unit leaf rates have been calculated from the dry weight data (see Tables 4 and 5), the first and third of these quantities being calculated from the equations

$$R = \frac{\log_e W_2 - \log_e W_1}{t_2 - t_1},$$

and

$$E = \frac{\log_e w_2 - \log_e w_1}{t_2 - t_1} \times \frac{W_2 - W_1}{w_2 - w_1},$$

where the subscripts 1 and 2 refer to the harvests between which the average value of the quantity is being calculated, and $w \equiv (W_L)$.

Statistical Treatment.

In the statistical treatment of the data we have had the valued advice of Mr. E. A. Cornish, who has described the methods adopted in an appendix. The results of the analyses of variance are given in the tables.

DISCUSSION.

The Growth Curves.

Treatment Effects.

The dry weight curves of the whole plants in Experiment 2 are presented in fig. 2, and those of the parts in both experiments in figs. 3 and 4. Certain characteristic effects of nitrogen treatment are obvious from these figures. In the following paragraphs are presented systematically some further conclusions drawn from the statistical examination of the data: I-IV signify plants of the various treatments, and the arabic figures the harvests.

Wheat.

First leaf. 2: IV almost significantly below II and significantly below I; thereafter insignificantly different from both II and III; 4: III significantly greater than I.

Third leaf. 3: depression insignificant; 4, 5, and 6 taken together: II and III each significantly greater than I; 7: no significant differences.

Total leaves. 2: I–III insignificantly different, IV significantly depressed; 3 and 4: IV insignificantly different from III; 6: depression in IV again significant; 8, 9, and 10 taken together: IV significantly greater than III.

Roots. 6: II and III significantly below I, IV significantly below the others; 7–10: IV insignificantly different from III.

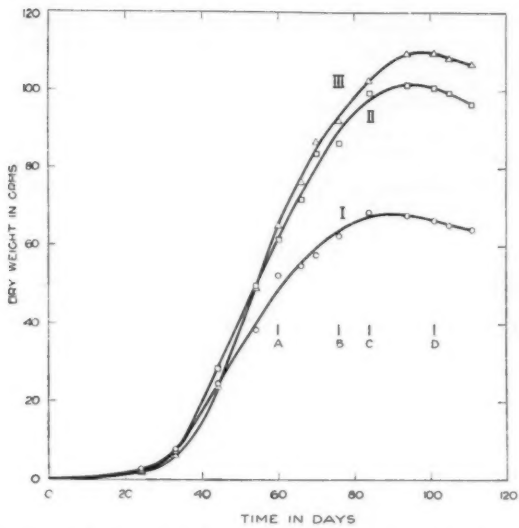

Fig. 1. Growth curves of oats with I, 0·4 gm. nitrogen; II, 0·8 gm.; and III. 1·2 gm. per pot. A, exsertion of inflorescences; B, flowering; C, grain in the pre-resting stage; D, grain in the resting stage. (From data of Blanck and Giesecke, 1934).

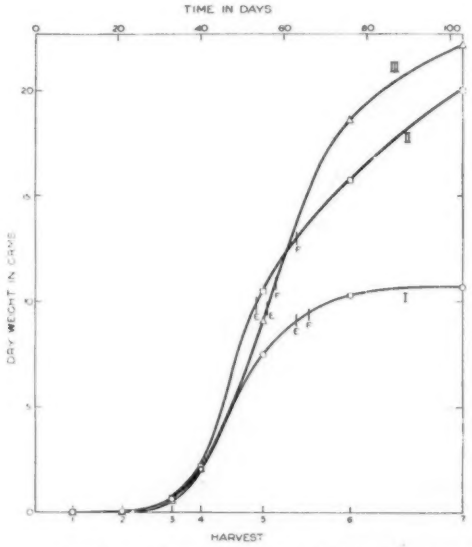

Fig. 2. Growth curves of Sudan grass with three nitrogen treatments, I, II, and III, Experiment 2. In this and subsequent figures, E indicates time of exsertion of inflorescences, and F time of flowering.

Stems. 2: I–III insignificantly different IV significantly depressed; 3 and 4 together: IV insignificantly different from III; 6 and 7–9 together: IV again significantly depressed; 10: III and IV insignificantly different.

Inflorescences. IV significantly greater than III, taking the data as a whole.

Total shoots. 2: IV significantly below III; 3 and 4: IV insignificantly different from III; 6: IV depressed again; thereafter insignificantly different from III.

Total plant. 6: IV significantly below II; IV never significantly greater than III.

Sudan grass.

Leaves. 2 and 3: III significantly depressed, I and II insignificantly different; 6 and 7: III significantly greater than II.

Roots. 2–5 taken together: III significantly depressed below II; III never becomes significantly greater than II.

Stems. 2–5 taken together: depression in III barely significant; 6–7 taken together: IV insignificantly greater than II.

Inflorescences. Treatment effect between each pair significant on the whole.

Total plant. 2–5 taken together: III significantly depressed below II; III never significantly greater than II.

With the highest treatment, vegetative organs and whole plants are depressed in size during adolescence, the roots more markedly than the leaves. Although the depression passes off, only the leaves and inflorescences become significantly greater in weight than those of the preceding treatment; and their excess weight is insufficient to affect significantly the weight of the whole plant. There is a suggestion in the wheat experiment that the depression wears off after harvest 2 and appears again at harvest 6: such a phenomenon could be produced by interaction of treatment and environment.

Two factors may contribute to the ultimate increase in leaf weight resulting from nitrogen treatment: individual leaves may attain a greater size, or a greater number of leaves may be formed. Probably both factors operate. Increased nitrogen supply tends slightly to increase the mature weight of the early individual leaves[4]; it also causes increased development of leaf primordia (cf. Crowther, 1934) and of tillers (see Table 6).

Drifts with Time.

In the wheat experiment, the roots show maximum weight at harvest 8, the leaves at harvest 8 with higher treatments and a little earlier with lower treatments. These maxima are approximately coincident with flowering. Thereafter a significant decrease in leaf weight occurs with all treatments[5], probably in part owing to breakdown of tissues consequent upon translocation of materials to the inflorescence: the absolute decrease is greater, the greater the inflorescence weight.

[4] Increase in size of leaves with high nitrogen treatment is a generally observed phenomenon (Mothes, 1932; Garner et al., 1934).

[5] A rather similar picture is given by the data of Blanck and Giesecke (1934), except that there the root maximum is delayed with increasing treatment.

The loss from the roots (insignificant in plants of treatment IV) may be due partly to decomposition.

In the Sudan grass the roots increase in weight to the end of the experiment,

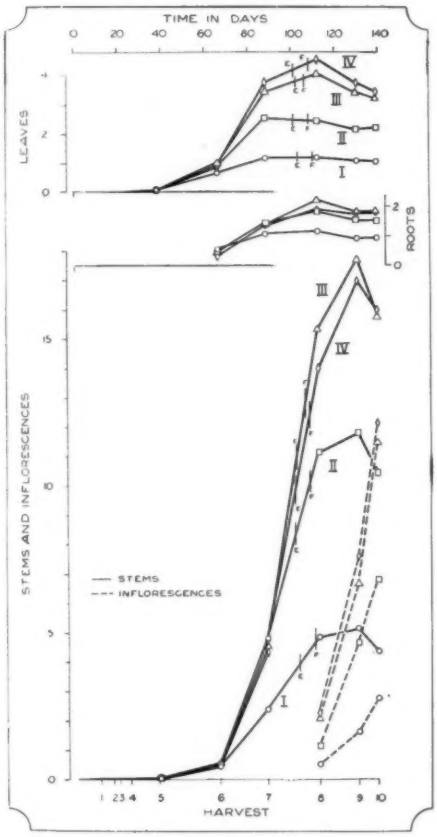

Fig. 3.

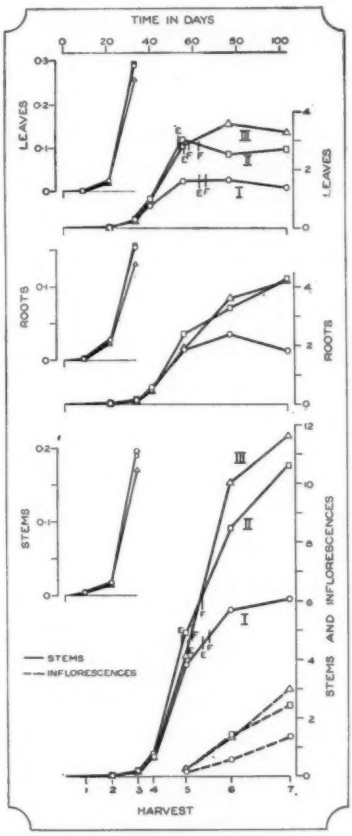

Fig. 4.

Fig. 3. Growth curves (dry weight in gm.) of parts in wheat, Experiment 1.

Fig. 4. Growth curves of parts in Sudan grass, Experiment 2. The early portions of the curves are repeated with enlarged scale of abscissae.

except those of treatment I, whose weight falls significantly. The leaves attain maximum weight approximately at flowering (later with treatment III), but there is no appreciable loss thereafter.

The final harvest in this experiment was made before the seeds had passed into the resting stage, and loss in leaf weight might have occurred later. Nevertheless the post-flowering period of growth is clearly expanded in Sudan grass as compared with wheat; this is probably due partly to the smaller inflorescences of Sudan grass and partly to the temperature, which fell during the senescence of the Sudan grass, and rose during that of the wheat.

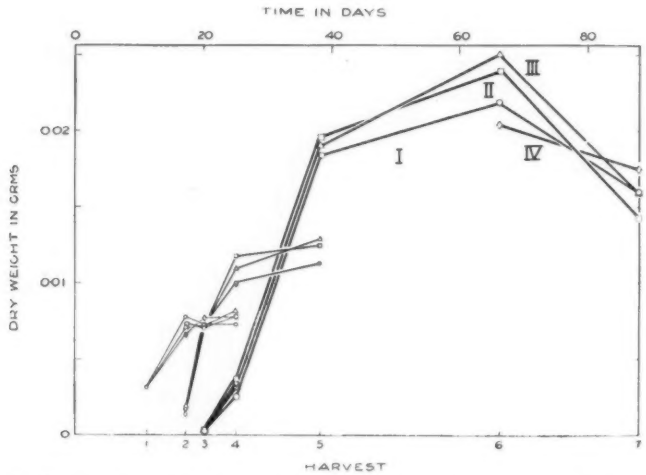

Fig. 5. Growth curves of first, second, and third leaves in wheat, Experiment 1.

The curves for the individual leaves of wheat are shown in fig. 5. It is seen that the early growth of each leaf is coincident with cessation of growth in the previous leaf, as was shown for the potato plant by Stone (1933). The data for the third leaf suggest that senescence may be retarded by increasing treatment.

Data of Other Investigators.

The effect of nitrogen on the growth of *Helianthus* has been observed by Rippel and Ludwig (1926), using ammonium nitrate; on that of oats by Meyer (1927) and Rippel and Meyer (1933), using ammonium sulphate, and by Blanck et al. (1933, 1934), using ammonium nitrate; on that of barley and wheat by Brenchley and Jackson (1921), using sodium nitrate; and on that of the sub-aerial portions of Sudan grass and barley by Ballard (1933), using sodium nitrate. The data of Blanck and Giesecke (1934) have been plotted in fig. 1.

All these investigations (except that of Brenchley and Jackson, where replication was poor) show that, after a certain point, increase of the initial nitrogen

supply depresses the growth in the early stages. The depression disappears with time and, up to some optimum value of nitrogen supply, determined by the conditions and the species, plants with the greatest supply attain the greatest size. A number of the features exhibited by the present data are also to be seen in the data of Crowther (1934) for the cotton plant.

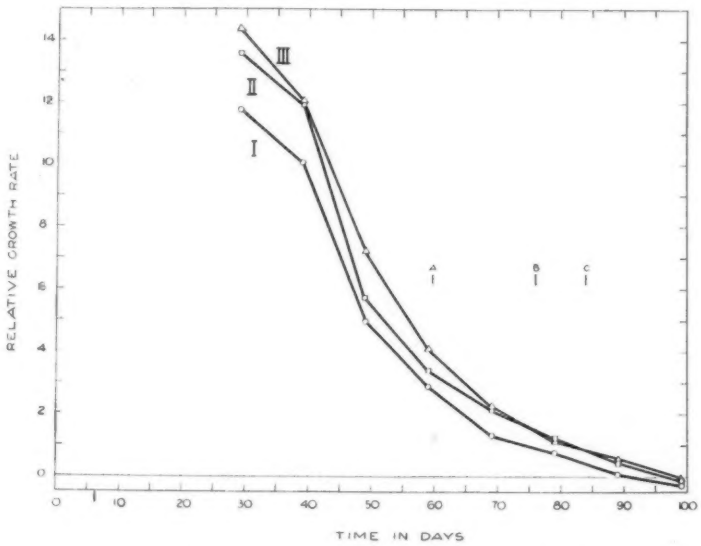

Fig. 6. Curves of relative growth rate (gm. per 100 gm. per day, i.e. $R \times 100$) of oats with three different nitrogen treatments. Calculated by interpolation in the curves of fig. 1. A, B, C, as in fig. 1.

Effect of Initial Depression on Final Weight of Plant.

The available data suggest that the initial depression prevents the full potentiality of a high nitrogen supply being manifested within the growing period. The depression appears to be due to the addition of a large amount of nitrogen to the external medium when the plant is very small. If the addition is distributed over a period during early growth, or if the main addition is made some time after the commencement of growth, the final size attained by the plant for a given amount of nitrogen supplied is greater, and the depression appears not to be manifested. Neidig and Snyder (1922) found that successive additions of sodium nitrate throughout the growing period of wheat gave yields in excess of those obtained when the total nitrate was applied at planting time. Gericke (1920, 1922) found that a greater yield of wheat was obtained when the main addition of nitrogen was

made from 33 to 110 days after planting than when it was made at the time of planting. This effect of delayed application, however, holds only up to a certain point in the growing period: Davidson and LeClerc (1917, 1918, 1923) found that application at the time of exsertion of inflorescences produced no increase in yield.

TABLE 2.

Mean Dry Weights in gm. per Plant for Experiment 1 (Wheat).

Harvest	Treatment. I		II		III		IV	
First Leaf								
1	0·00306		0·00306		0·00306		0·00306	
2	0·00778		0·00727		0·00711		0·00656	
3	0·00732		0·00716		0·00731		0·00765	
4	0·00729		0·00786		0·00823		0·00778	
Analysis of variance performed on unchanged dry weight data, excluding harvest 1. Treatment as a whole insignificant, but interaction and time significant.								
S.E. for means of 5 = ±0·000248.								
S.E. for means of 6 = ±0·000320.								
Second Leaf								
2	0·00172		0·00164		0·00189		0·00138	
3	0·00715		0·00656		0·00655		0·00412	
4	0·0100		0·0117		0·0111		0·00987	
5	0·0114		0·0125		0·0129		—	
Third Leaf								
3	0·00025	±0·00008	0·00030	±0·00004	0·00027	±0·00005	0·00021	±0·00007
4	0·00247	±0·00019	0·00353	±0·00026	0·00318	±0·00020	0·00345	±0·00045
5	0·0197	±0·0008	0·0216	±0·0012	0·0206	±0·0013	—	—
6	0·0219	±0·0019	0·0245	±0·0016	0·0251	±0·0008	0·0204	±0·0023
7	0·0159	±0·0008	0·0143	±0·0008	0·0159	±0·0005	0·0174	±0·0017
Interaction insignificant. Standard errors of means given.								
Total Leaves								
1	0·00306		0·00306		0·00306		0·00306	
2	0·00950	*0·977*	0·00891	*0·949*	0·00899	*0·953*	0·00793	*0·894*
3	0·0147	*1·166*	0·0140	*1·145*	0·0141	*1·150*	0·0147	*1·166*
4	0·0198	*1·297*	0·0231	*1·362*	0·0226	*1·354*	0·0211	*1·323*
5	0·0640	—	0·0692	—	0·0811	—	—	—
6	0·664	*2·822*	0·936	*2·971*	0·989	*2·994*	0·857	*2·932*
7	1·17	*3·066*	2·53	*3·403*	3·43	*3·535*	3·75	*3·573*
8	1·19	*3·075*	2·45	*3·388*	4·01	*3·603*	4·47	*3·649*
9	1·08	*3·032*	2·15	*3·333*	3·40	*3·531*	3·73	*3·572*
10	1·07	*3·030*	2·20	*3·343*	3·20	*3·504*	3·42	*3·532*
Harvest 5 omitted from analysis of variance.								
S.E. for means of 5 = ±*0·0140*.								
S.E. for means of 3 = ±*0·0181*.								
Roots								
6	0·527	*2·706*	0·425	*2·619*	0·415	*2·616*	0·289	*2·453*
7	1·04	*3·018*	1·41	*3·148*	1·33	*3·122*	1·32	*3·123*
8	1·15	*3·057*	1·77	*3·247*	2·18	*3·337*	1·86	*3·266*
9	0·885	*2·946*	1·50	*3·176*	1·82	*3·258*	1·73	*3·235*
10	0·889	*2·947*	1·50	*3·171*	1·82	*3·258*	1·74	*3·240*
S.E. for means of 6 = ±*0·0260*.								
S.E. for mean of 5 = ±*0·0335*.								

TABLE 2 (cont.).

Harvest	Treatment I		Treatment II		Treatment III		Treatment IV	
Stems								
1	0·00182		0·00182		0·00182		0·00182	
2	0·00321	*0·505*	0·00305	*0·484*	0·00322	*0·505*	0·00252	*0·396*
3	0·00309	*0·489*	0·00318	*0·501*	0·00343	*0·533*	0·00333	*0·522*
4	0·00556	*0·743*	0·00679	*0·829*	0·00643	*0·808*	0·00351	*0·765*
5	0·0271	—	0·0298	—	0·0338	—	—	—
6	0·425	*2·623*	0·529	*2·722*	0·570	*2·753*	0·486	*2·685*
7	2·38	*3·376*	4·80	*3·689*	4·52	*3·655*	4·32	*3·634*
8	4·85	*3·685*	11·13	*4·046*	15·33	*4·184*	13·97	*4·141*
9	5·14	*3·710*	11·81	*4·072*	17·71	*4·248*	16·96	*4·229*
10	4·36	*3·640*	10·44	*4·018*	15·76	*4·197*	15·97	*4·202*
	Harvest 5 omitted from analysis of variance.							
	S.E. for means of 5 = ±*0·0162*.							
	S.E. for means of 3 = ±*0·0209*.							
Inflorescences								
8	0·530	±0·020	1·41	±0·05	2·03	±0·06	2·23	±0·08
9	1·62	±0·11	4·63	±0·24	6·67	±0·39	7·61	±0·38
10	2·77	±0·05	6·80	±0·13	11·47	±0·35	12·13	±0·27
Mean for all harvests		*3·123*		*3·548*		*3·729*		*3·770*
	Interaction insignificant; treatment significant.							
	S.E. for treatment means (of 15) = ±*0·0104*.							
	S.E.'s of individual dry weight means given.							
Primary Shoot								
1	0·00488		0·00488		0·00488		0·00488	
2	0·0127		0·0120		0·0122		0·0104	
3	0·0177		0·0172		0·0176		0·0181	
4	0·0254		0·0299		0·0289		0·0270	
5	0·0738		0·0830		0·0888		—	
6	0·466		0·554		0·556		0·516	
7	1·76		2·40		2·15		2·09	
8	3·36		4·73		5·10		4·88	
9	4·39		5·87		6·33		6·03	
10	4·30		6·33		6·23		6·41	
Total Shoots								
1	0·00488		0·00488		0·00488		0·00488	
2	0·0127	*1·103*	0·0120	*1·095*	0·0122	*1·086*	0·0104	*1·013*
3	0·0177	*1·248*	0·0172	*1·234*	0·0176	*1·244*	0·0181	*1·255*
4	0·0254	*1·403*	0·0299	*1·474*	0·0289	*1·462*	0·0270	*1·430*
5	0·0898	—	0·0990	—	0·1147	—	—	—
6	1·08	*3·035*	1·47	*3·165*	1·55	*3·191*	1·34	*3·127*
7	3·55	*3·549*	7·33	*3·865*	7·94	*3·900*	8·07	*3·906*
8	6·57	*3·817*	14·99	*4·175*	21·37	*4·329*	20·66	*4·313*
9	7·83	*3·894*	18·59	*4·269*	27·78	*4·444*	28·31	*4·451*
10	8·20	*3·914*	19·44	*4·289*	30·42	*4·483*	31·53	*4·498*
	Harvest 5 omitted from analysis of variance.							
	S.E. for means of 5 = ±*0·0143*.							
	S.E. for means of 3 = ±*0·0185*.							
Total Plant								
6	1·62	*3·206*	1·89	*3·275*	1·97	*3·295*	1·63	*3·210*
7	4·59	*3·662*	8·74	*3·941*	9·28	*3·967*	9·40	*3·972*
8	7·71	*3·887*	16·76	*4·224*	23·54	*4·371*	22·52	*4·350*
9	8·72	*3·940*	20·09	*4·303*	29·60	*4·471*	30·04	*4·479*
10	9·09	*3·958*	20·93	*4·321*	32·24	*4·508*	33·26	*4·522*
	S.E. for means of 5 = ±*0·0132*.							
	S.E. for mean of 3 = ±*0·0170*.							

NOTES.

The means of the logarithms of the dry weight data and their standard errors are in italic figures. All values are means of 5, except treatment IV, harvests 3, 4, and 6, which are means of 3.

Also the increase in yield obtained by delayed application may be dependent upon no other nutrients being limiting.

Garner et al. (1934) found with *Nicotiana tabacum* that 80 lb. nitrogen per acre applied initially in one dose gave an early retardation in growth and no greater yield than a dose of 40 lb.; but if half the quantity was applied at the commencement of growth and the other half 30 days later, a greater yield was obtained, and retardation was not evident. Somewhat similar effects were found in oats by Rackmann (1935), except that in this case the doses comprised nitrogen, phosphorus, and potassium.

The Relative Growth Rate.

Relative growth rates for the two experiments are presented in Tables 4 and 5 and in figs. 7 and 8. Values have been calculated from the data of Blanck and Giesecke also, by interpolating in the smoothed growth curves[6] of fig. 1; these are presented graphically in fig. 6. The following conclusions can be drawn from statistical examination of the data of the present experiments.

Wheat.

6–7: II and III significantly greater than I; IV significantly greater than III; 6–7 to 8–9 taken together: IV insignificantly greater than III; otherwise significant increases with treatment from 6–7 to 8–9; 9–10: no significant differences.

Sudan grass.

1–2: III significantly less than I; 2–3 to 4–5: II and III insignificantly different; 2–3 to 6–7: I and II insignificantly different, even if 3–4 omitted; 5–6: III significantly greater than II.

Evidently, owing to the insensitivity of the statistical method, in some places it has not been possible to show significant differences between relative growth rates where such differences must have occurred. Thus, at some time, R for the Sudan grass plants of treatment II must have been greater than R for those of treatment I, although we cannot conclude this from the R data.

All the curves presented, however, conform to the same general picture. In the early stages, R is less the greater the nitrogen supply; this holds presumably only beyond the level of supply at which depression becomes evident. After the maximum is reached, R is higher with the higher treatments than with the lower treatments. The curves tend to converge at the end of the growing period.

Relative growth rate curves calculated from the other data referred to in the previous section tend to conform to the general picture, although certain of them exhibit irregularities probably due to the variability of the material.

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[6] Briggs, Kidd, and West found a depression in R immediately before flowering. This may have been eliminated by the smoothing method adopted in the present case, but the method enables us to depict the general trend of the effect of treatment. This depression would not be exhibited in the experimental data of the present paper owing to the large intervals between harvests.

Leaf Weight Ratio.

Among the data so far considered, leaf weight ratios can be calculated only from those presented in this paper. The values are given in Tables 4 and 5, and are plotted in figs. 9 and 10. The Sudan grass curves show no significant effect of

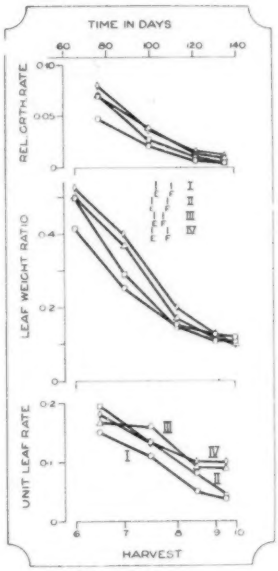

Fig. 7.

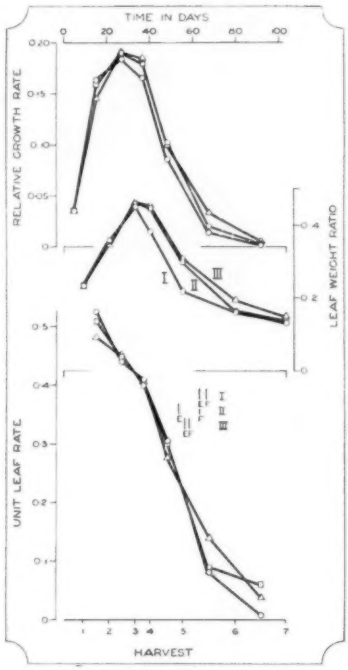

Fig. 8.

Fig. 7. Curves of relative growth rate (gm. per gm. per day), leaf weight ratio and unit leaf rate (gm. per gm. per day) in wheat, Experiment 1.

Fig. 8. Curves of relative growth rate (gm. per gm. per day) leaf weight ratio and unit leaf rate (gm. per gm. per day) in Sudan grass, Experiment 2.

treatment until after the maxima have been attained, when the ratio becomes an increasing function of treatment, and this is the state exhibited in the curves for wheat[7]. At the end of the growing period, the curves converge, becoming insignificantly different at the final harvest.

[7] The results of Müller and Larsen (1935) for *Sinapis alba* also show that increased nitrogen content of leaves is associated with increase in F_A.

TABLE 3.

Mean Dry Weights in gm. per Plant for Experiment 2 (Sudan Grass).

Harvest	Treatment I		Treatment II		Treatment III	
Leaves						
1	0·0023		0·0023		0·0023	
2	0·0251	*1·397*	0·0234	*1·369*	0·0199	*1·294*
3	0·288	*2·455*	0·293	*2·465*	0·258	*2·407*
4	0·773	*2·877*	1·000	*2·988*	0·928	*2·965*
5	1·63	*3·210*	3·05	*3·471*	2·82	*3·446*
6	1·65	*3·218*	2·56	*3·406*	3·61	*3·557*
7	1·39	*3·140*	2·72	*3·430*	3·30	*3·514*
	S.E. for means of 6 = ±*0·0149*.					
	S.E. for means of 5 = ±*0·0164*.					
Roots						
1	0·0032		0·0032		0·0032	
2	0·0282	*1·447*	0·0258	*1·406*	0·0225	*1·350*
3	0·154	*2·182*	0·157	*2·193*	0·131	*2·116*
4	0·597	*2·768*	0·507	*2·693*	0·465	*2·661*
5	1·87	*3·269*	2·40	*3·369*	1·93	*3·283*
6	2·37	*3·375*	3·29	*3·513*	3·63	*3·558*
7	1·81	*3·230*	4·28	*3·618*	4·24	*3·618*
	S.E. for means of 6 = ±*0·0401*.					
	S.E. for means of 5 = ±*0·0440*.					
Stems						
1	0·0044		0·0044		0·0044	
2	0·0169	*1·227*	0·0168	*1·224*	0·0148	*1·168*
2	0·197	*2·288*	0·190	*2·278*	0·171	*2·228*
4	0·682	*2·820*	0·738	*2·846*	0·660	*2·816*
5	3·82	*3·582*	4·90	*3·663*	4·11	*3·613*
6	5·68	*3·753*	8·48	*3·928*	10·01	*4·000*
7	6·07	*3·778*	10·61	*4·017*	11·62	*4·062*
	S.E. for means of 6 = ±*0·0338*.					
	S.E. for means of 5 = ±*0·0371*.					
Inflorescences						
5	0·158		0·196		0·246	
6	0·580		1·42		1·35	
7	1·37		2·44		3·00	
Mean for all harvests		*2·137*		*3·011*		*3·321*
	Interaction insignificant. Treatment significant.					
	S.E. for treatment means (of 15) = ±*0·0869*.					
Total Plant						
Seed	0·00727		0·00727		0·00727	
1	0·0099		0·0099		0·0099	
2	0·0702	*1·845*	0·0660	*1·819*	0·0572	*1·755*
3	0·639	*2·801*	0·641	*2·805*	0·560	*2·745*
4	2·05	*3·305*	2·25	*3·331*	2·05	*3·309*
5	7·49	*3·873*	10·52	*4·006*	9·11	*3·958*
6	10·29	*4·012*	15·75	*4·197*	18·61	*4·269*
7	10·64	*4·022*	20·04	*4·298*	22·16	*4·343*
	S.E. for means of 6 = ±*0·0279*.					
	S.E. for means of 5 = ±*0·0306*.					

NOTES.

The means of the logarithms of the dry weight data and their standard errors are in italic figures. Values for harvests 1–4 are means of 6; and for harvests 5–7, means of 5.

Figs. 11 and 12 are of interest in connection with these drifts. They show that, during adolescence, roots and leaves constitute most of the plant; later the stems, and finally the inflorescences, become fractions of increasing relative magnitude. Three successive phases of ontogeny are thus manifested, in which growth of leaves and roots, stems, and inflorescences respectively preponderate.

TABLE 4.

Relative Growth Rate, Unit Leaf Rate, and Leaf Weight Ratio in Experiment 1 (Wheat).

Harvest	Treatment I	II	III	IV
Relative Growth Rate ($100R$)				
6–7	4·73 ± 0·280	6·96 ± 0·225	7·06 ± 0·196	7·96 ± 0·357
7–8	2·16 ± 0·100	2·71 ± 0·125	3·88 ± 0·167	3·61 ± 0·261
8–9	0·68 ± 0·050	1·01 ± 0·130	1·28 ± 0·212	1·64 ± 0·327
9–10	0·46 ± 0·109	0·46 ± 0·230	0·95 ± 0·267	1·13 ± 0·409
Leaf Weight Ratio ($100F_W$)				
6	41·5	49·7	50·1	52·6
7	25·4	29·0	37·0	40·0
8	15·4	14·6	17·1	20·0
9	12·4	10·7	11·5	12·5
10	11·8	10·5	9·9	10·3

All values are means of 5, except that for harvest 6, treatment IV, which is a mean of 3.

S.E. for means of 5 = ±0·66.
S.E. for mean of 3 = ±0·85.

Unit Leaf Rate ($100E_W$)				
6–7	15·11 ± 0·751	19·42 ± 0·706	16·93 ± 0·546	18·02 ± 0·945
7–8	11·05 ± 0·480	13·41 ± 0·698	16·00 ± 0·973	13·33 ± 1·263
8–9	4·95 ± 0·358	8·05 ± 0·992	9·11 ± 1·485	10·22 ± 1·974
9–10	3·82 ± 0·922	4·29 ± 2·192	8·89 ± 2·543	10·02 ± 3·599

Since it will be shown in the next section that treatment does not increase E_W during early adolescence, its effect on F_W is evidently due firstly to one on the distribution of synthesized materials among the parts. The initial depression, possibly a toxic effect, is more prolonged in roots than in leaves; this presumably leads to the first increase with treatment in F_W. Immediately F_W is increased, the relative rate of dry matter production is increased, and a greater proportion of the relative excess dry matter produced with high treatment is used for leaf growth than for the other parts. This is responsible for further increase of leaf weight ratio and decrease of root weight ratio with treatment[8]. The effect on F_W becomes apparent in the data after the third harvest, when the growth curves of the leaves

[8] Decrease in root weight ratio with increasing nitrogen supply was also shown by Turner (1922), Mothes (1932), and Rippel and Meyer (1933), and can be seen in the data of Brenchley and Jackson (1921).

begin to diverge. The effect on the stem weight ratio is intermediate between those on root and leaf weight ratios; and inflorescence weight ratio, although increased in wheat, does not appear to be significantly affected in the Sudan grass experiment up to the time of the final harvest.

TABLE 5.

Relative Growth Rate, Unit Leaf Rate, and Leaf Weight Ratio in Experiment 2 (Sudan Grass).

Harvest	Treatment I	Treatment II	Treatment III
Relative Growth Rate ($100R$)			
Sowing to harvest 1	3·45 ± 0·985	3·45 ± 0·985	3·45 ± 0·985
1–2	16·31 ± 0·315	15·79 ± 0·246	14·60 ± 0·444
2–3	18·40 ± 0·676	18·94 ± 0·424	19·01 ± 0·602
3–4	16·65 ± 1·520	17·94 ± 1·558	18·54 ± 1·061
4–5	8·64 ± 0·569	10·28 ± 0·753	9·94 ± 0·487
5–6	1·51 ± 0·192	1·92 ± 0·261	3·40 ± 0·243
6–7	0·12 ± 0·254	0·89 ± 0·255	0·65 ± 0·211
Leaf Weight Ratio ($100F_W$)			
1	23·4	23·4	23·4
2	35·8	35·6	34·6
3	45·1	45·7	46·0
4	38·1	44·7	45·3
5	21·9	29·6	30·9
6	16·1	16·2	19·4
7	13·1	13·6	14·9

Values for harvests 2–4 are means of 6; of harvests 5–7, means of 5.

S.E. for means of 6 = ±0·87.
S.E. for means of 5 = ±0·96.

Unit Leaf Rate ($100E_W$)			
1–2	52·47 ± 2·625	51·24 ± 7·918	48·14 ± 20·555
2–3	43·99 ± 3·958	44·85 ± 2·785	45·12 ± 9·302
3–4	41·08 ± 5·134	39·8 ± 0·614	40·67 ± 3·520
4–5	31·46 ± 2·216	29·99 ± 2·494	27·67 ± 2·169
5–6	8·13 ± 1·054	8·90 ± 1·069	14·14 ± 0·960
6–7	0·85 ± 2·359	6·02 ± 1·922	3·81 ± 1·341

Various factors contribute to the convergence during senescence. Leaf growth ceases at about the time of flowering, and in wheat the dry weights of leaves actually converge in senescence. Root growth continues later in Sudan grass in the higher treatments, causing crossing in the root weight ratio curves, and this obviously contributes to convergence in the F_W curves. Finally in wheat the inflorescence weight ratio increases with treatment. The loss in weight of the leaves is probably associated with the high inflorescence weight ratio of this plant, since in the Sudan grass the loss is inappreciable.

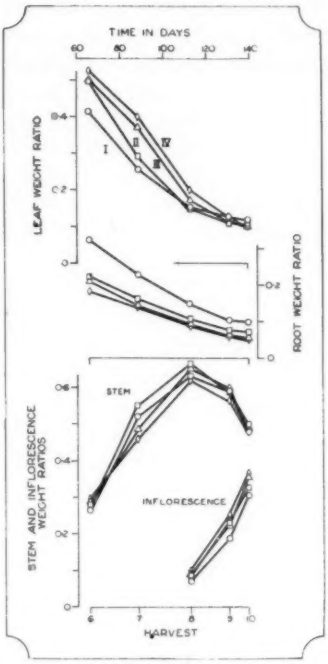

Fig. 9.

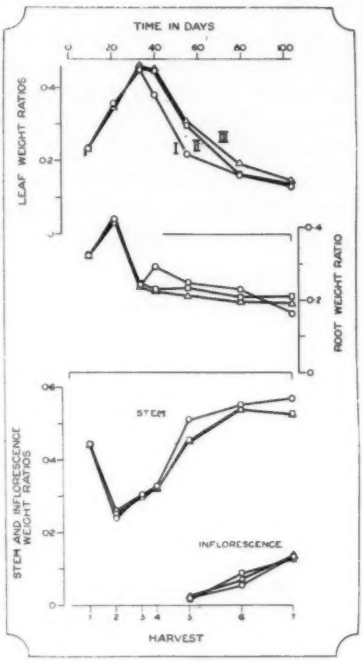

Fig. 10.

Fig. 9. Curves of ratios of parts to whole plant in wheat, Experiment 1.
Fig. 10. Curves of ratios of parts to whole plant in Sudan grass, Experiment 2.

The Unit Leaf Rate.

Unit leaf rates are presented in Tables 4 and 5 and figs. 7 and 8. The values are slightly in error on account of the exclusion of the leaf sheaths, which perform a certain amount of carbon assimilation; the error is less the younger the plant. The following conclusions have been drawn from statistical examination of the data.

Wheat.

6–7: III significantly below II; III and IV insignificantly different; 6–7 to 8–9: I significantly below II at each point; 7–8 and 8–9: II, III, and IV insignificantly different; 9–10: no significant differences.

Sudan grass.

5–6: III significantly greater than II; no other differences demonstrably significant.

The effect of treatment on E_W has clearly differed in the two experiments. In the wheat the effect is evident between plants of treatment I and the others, at least from a point very soon after harvest 6, at which time the leaves as a whole are still adolescent. In the Sudan grass there is no treatment effect except for a brief period after flowering. Depression is shown with treatment III between harvests 6 and 7 in the wheat experiment; it is not shown with treatment IV, but the value of E_W is here somewhat unreliable on account of fewness of replicates. There is no significant depression in the Sudan grass experiment.

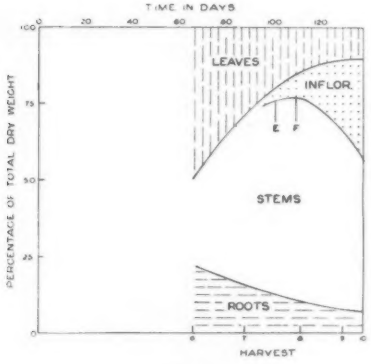

Fig. 11.

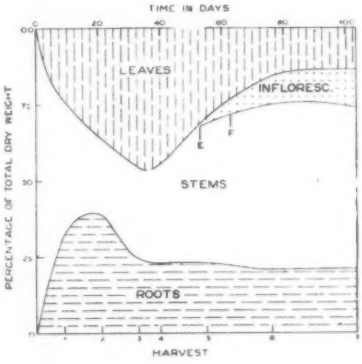

Fig. 12.

Fig. 11. Diagram showing the relative proportions of parts in wheat during the latter half of the growing period, Experiment 1, treatment II. The conventions of separation in this and the next figure are those described in the text.

Fig. 12. Diagram showing the relative proportions of parts in Sudan grass during the growing period, Experiment 2, treatment II. The ungerminated seed has been arbitrarily classed as stem.

Gregory (1926) has presented graphically values for E_A for plants with two different nitrogen supplies. The observations ceased with attainment of maximum leaf area, which approximately coincides with flowering. Gregory concludes that difference in nitrogen supply has had no effect on E_A up to that time. Crowther (1934) also finds that nitrogen treatment has no effect on E_W during the main growth phase of the cotton plant[9]. These conclusions agree essentially with the results of the Sudan grass experiment, but not with those of the wheat.

Gregory also concludes that, up to the time of attainment of maximum leaf area, E_A is independent of the age of the plant, and that the fall with time is due to environmental changes. Observations on the environment during the course of the present experiments render it very improbable that the marked decline in

[9] Crowther omitted the root weights in the calculation of his values of E_W.

E_W was due to environmental factors. The Sudan grass senesced during conditions of falling temperature, and the wheat during conditions of rising temperature.

Since the data presented in this paper show that during part of the life-period E_W can be increased by nitrogen treatment, further analysis of this phenomenon is desirable. There are two possibilities: treatment may increase the rate of carbon assimilation of the individual leaves at a given growth stage, or it may decrease the average rate of ontogeny of the leaves as a whole, and so prolong their effective life. The latter could be accomplished either by inducing a longer life of individual leaves, or by causing a prolonged formation of young leaves.

TABLE 6.

Mean Number of Tillers per Plant for Experiment 1 (Wheat).

	Treatment			
Harvest	I	II	III	IV
Total Tillers				
6	4·4	6·5	6·9	6·3
7	4·1	6·5	7·9	8·9
8	3·9	6·9	7·9	7·5
9	4·3	6·2	7·4	8·4
10	4·0	6·5	8·6	8·2

All values are means of 5 except that for harvest 6, treatment IV, which is a mean of 3.

S.E. for means of 5 = ±0·06.
S.E. for mean of 3 = ±0·08.

Inflorescence-bearing Tillers				
8	2·3	3·7	5·7	5·9
9	2·1	3·8	5·8	6·5
10	2·2	3·7	6·2	6·4

Interaction insignificant.

With regard to the first possibility, Gregory and Richards (1929) found that nitrogen deficiency lowered the rate of carbon assimilation of mature leaves at high light intensity, but the decrease was insignificant. This result, however, was obtained with high carbon dioxide concentration. Müller (1932) and Müller and Larsen (1935) found that the assimilation rate of *Sinapis alba* per unit leaf area increased with nitrogen supply and nitrogen content of the leaf; the same was found by Gassner and Goeze (1934 *a* and *b*) in wheat and rye, provided the supply of potassium was not low. The first possibility suggested is therefore probably a contributing factor.

To obtain information about the second possibility, the separation of the primary shoot and early leaves was performed in the wheat experiment. The data do not show that increasing nitrogen supply increases the longevity of the primary

shoot. Fairfield Smith (1933) and Bartel (1935) have shown that tillers are not individual units, but that competition between them for elaborated materials may occur, and this may account for the result obtained.

The data of the wheat experiment suggest that the third leaves decline in weight less rapidly with treatment IV than with the other treatments. Gassner and Goeze (1934*b*) also showed that the decline with age in rate of assimilation occurs later in leaves with high nitrogen content. Such an effect on rate of ontogeny of the individual leaf could be a factor contributing to the effect of treatment on E. It is, however, unjustifiable to extend this conclusion to the leaves at the end of the growing period of the plant: other elements such as phosphorus may become limiting in the larger plants earlier than in the smaller ones, and this might even hasten senescence in the late-formed leaves.

From Table 6 it can be seen that, whereas plants of treatments I and II had ceased to tiller before harvest 6, those of the higher treatments had not ceased till some weeks later; and it is most likely that the increased unit leaf rates of treatments III and IV towards the end of the life-period are due partly to the active assimilation of the young leaves of these later-formed tillers.

F_W AND E_W AS DETERMINANTS OF R.

Reviewing the conclusions drawn from this discussion, we see that the data do not permit us to state definitely whether both these factors contribute to the early depression in R with increasing nitrogen treatment, although insignificant depressions are shown in both. In the wheat experiment, both factors contribute to an increase in R with treatment throughout a considerable part of the growing period, although the effect of E_W is shown only between treatments I and II; in the Sudan grass experiment, the effect of F_W is the main one, and that of E_W is negligible until after the exsertion of inflorescences.

ONTOGENETIC RATE.

Needham (1933) has shown that in the development of an animal embryo the ontogenetic processes can be dissociated experimentally. The dissociation may, however, be only partial, so that a certain factor may affect the rate of different processes in different ways.

In the present experiments low nitrogen supply caused late exsertion of inflorescences and flowering, although in the Sudan grass experiment this conclusion is based on too few data to be reliable. Garner et al. (1934) showed that low and also very high nitrogen supply retarded flowering in the tobacco plant. On the other hand, Borodin (1931) found that in extreme nitrogen deficiency the appearance of inflorescences was earlier in barley and unaffected in time in millet.

Nitrogen treatment in the present experiments affected other processes differently: it delayed the decline of leaf weight ratio and hence of relative growth rate,

although it hastened flowering. Retardation in the development of vegetative parts was also shown by Gregory (1926) as a result of increasing nitrogen treatment.

SUMMARY.

This paper represents the first of a series devoted to the study of physiological ontogeny, and defines the effect of increasing the initial nitrogen supply to certain plants over a certain range.

It is found in general that, in the whole plant and its vegetative parts, increasing supply causes at first a depression in dry weight; later, this passes off and, provided the supply does not exceed a certain limit, gives place to an increase. In the early stages of growth, the relative growth rate is less the greater the nitrogen supply. After the maximum is attained the rate becomes greater with higher treatment. Similar phenomena are exhibited in the leaf weight ratio, only the depression could not be shown to be significant. The values of the ratio converge during senescence. The unit leaf rate was found to decline throughout practically the whole of the growing period, and no significant depression with treatment could be demonstrated. Increase with treatment was found in the later part of the growing period, and may be quite absent in the early part.

Other aspects of the change in weight of the plant and its parts are discussed.

STATISTICAL APPENDIX

by E. A. Cornish.

Before proceeding to the details of the methods employed in the statistical reduction of the data, it is necessary to remark upon the choice of a suitable variable for use in the analysis.

The analysis of variance and the z test are required here for the purpose of testing jointly the significance of the differences between several means, but the test is, strictly speaking, only applicable if the variance of a set of replicates is independent of the mean of these replicates.

An examination of the observational data indicated, in the majority of cases, the presence of a positive correlation between the treatment-harvest class (hereafter called class) mean and the class variance; and the following figures, which have been derived from an analysis of total shoots, Experiment 1, are fairly representative of the existing conditions. There was a significant positive correlation of $0{\cdot}75$ between the mean and the variance, while the correlation between the variance and the square of the class mean was $+0{\cdot}32$, a value which just failed to reach the 5 p.c. level of significance. It thus appeared that the square roots of the observations would provide a suitable variable. In this case, however, the coefficient had the value $+0{\cdot}62$, which, though indicating a weaker relationship, was nevertheless significant. The correlation, therefore, had not been eliminated and,

in a further endeavour to do this, the logarithms of the observations were tried. On evaluation this correlation was found to be —0·44, a significant value, showing that an over-correction had now been introduced. The regression of variance on mean was, however, relatively small, and in consequence it was decided to employ the logarithms where a change of variable was necessary. The correct unit, which may be found by a process of trial and error, would be some positive power of the observations less than $\frac{1}{2}$. In order to avoid negative values the dry weights were multiplied by 10^3 before transformation to logarithms.

TABLE 7.

Analysis of Variance. Total Shoots, Experiment 1 (grams).

Variation due to	Deg. freed.	Sum. sq.	Mean sq.
Between classes	34	17906·47	
Within classes	134	33·70	0·25152
Total	168	17940·17	

The following alternative test demonstrates, perhaps more forcibly, the differential variability of the classes. Working with the original weights in grams, the analysis of the total variability gave the results shown in Table 7.

If v_r $(r = 1, 2, \ldots\ldots, 35)$ stand for the variance of class r, then the mean variance, $\bar{v}$, is given by

$$\bar{v} = S(v)/35 = 2{\cdot}2577,$$

and the variance of variance, V, by

$$V = S(v - \bar{v})^2/34 = 891{\cdot}62 \quad (34 \text{ deg. freed}).$$

If there are no significant differences in variability among the several classes, the observed variances will be distributed about the weighted mean variance, 0·25152, with variance, v_0, given by

$$v_0 = 2(\cdot 25152^2)/(n-1) = 2(\cdot 25152^2)/3{\cdot}7488 = 0{\cdot}033751 \quad (134 \text{ deg. freed.}),$$

where n is the number of replicates per class. This number varied from class to class, and since the variance is inversely proportional to the number of observations, n has been taken as the harmonic mean of the class numbers. V and v_0 were compared by means of the z test, which demonstrated a significant differential variability among the classes.

METHODS OF ANALYSIS.

In the majority of cases, the experimental observations are non-orthogonal, and this fact necessitates a modification of the ordinary analytic procedure of the

analysis of variance in their treatment. Several methods are available for the analysis of non-orthogonal data, of which the most general in its range of application is the fitting of constants by the method of least squares, the remaining methods being applicable only under certain conditions. For a full discussion of the subject, recourse should be made to an excellent paper by Yates (1933), who devised the methods.

TABLE 8.

Class Totals for Third Leaf, Experiment 1.

	Treatment				
Harvest	I	II	III	IV	Total.
3	1·5977 (5)	2·3051 (5)	2·0053 (5)	0·8195 (3)	6·7276 (18)
4	6·9344 (5)	7·7157 (5)	7·4977 (5)	4·5950 (3)	26·7428 (18)
5	11·4611 (5)	11·6625 (5)	11·5476 (5)	—	34·6712 (15)
6	11·6635 (5)	11·9187 (5)	11·9908 (5)	6·9151 (3)	42·4881 (18)
7	10·9983 (5)	10·7653 (5)	11·0040 (5)	11·1652 (5)	43·9328 (20)
Total	42·6550 (25)	44·3673 (25)	44·0454 (25)	23·4948 (14)	154·5625 (89)

Figures in brackets indicate the number of observations contributing to the corresponding total.

TABLE 9.

Analysis of Variance.

Variation due to	Deg. freed.	Sum. sq.	Mean sq.
Constants t and h	7	50·8277	
Residual (interaction)	11	0·1345	0·01223
Between classes	18	50·9622	
Within classes	70	0·8579	0·01226
Total	88	51·8201	

TABLE 10.

Analysis of Variance.

Variation due to	Deg. freed.	Sum. sq.	Mean sq.
Harvest	4	3·28052	0·82013
Treatment	3	0·49413	0·16471
Residual (interaction)	12	0·12673	0·01056
Total	19	3·90138	

(1) *Fitting Constants.*

The observations on the third leaf, Experiment 1, are given in Table 8. As one class is entirely missing, these data must be analysed by fitting constants. The main purpose of the analysis is to establish the existence or otherwise of the inter-

action of treatment and harvest. On the hypothesis of negligible interaction, constants representing treatment and harvest are fitted, and the residual variance between classes, after fitting, is compared with the intraclass variance by means of the z test.

The preliminary analysis of variance gives the following results:

Total sum sq. $= 51 \cdot 8201$ (88 deg. freed.)
Between classes $= 50 \cdot 9622$ (18 deg. freed.)
Within classes $= 0 \cdot 8579$ (70 deg. freed.).

The constants to be determined are:

a general mean	m;
four treatment constants	t_1, t_2, t_3, t_4;
five harvest constants	h_1, h_2, h_3, h_4, h_5.

The solution of the normal equations results in the following values for the constants:

$m = +1 \cdot 744277$	$h_1 = -1 \cdot 371619$
$t_1 = -0 \cdot 038075$	$h_2 = -0 \cdot 259664$
$t_2 = +0 \cdot 030415$	$h_3 = +0 \cdot 563843$
$t_3 = +0 \cdot 017539$	$h_4 = +0 \cdot 615075$
$t_4 = -0 \cdot 009879$	$h_5 = +0 \cdot 452363$,

whence, the reduction in the sum of squares due to fitting constants, excluding the mean, is

$$1 \cdot 744277 \times 154 \cdot 5625 + (-0 \cdot 038075 \times 42 \cdot 6550) + \ldots\ldots + (-1 \cdot 371619 \times 6 \cdot 7276) + \ldots\ldots - 154 \cdot 5625^2/89 = 50 \cdot 8277.$$

The complete analysis of variance is given in Table 9.

It will be observed that all the variation between class means, except for a very small fraction, may be accounted for as the additive effect of treatment and harvest.

(2) *Approximate Analysis of Variance.*

In the remaining non-orthogonal data of Experiment 1, the class numbers do not depart appreciably from equality, so that their reduction has been effected by the method of unweighted means, an approximate analysis of variance, due to Yates. The details of this method are illustrated in the following analysis of total plant, Experiment 1. The class means are given in Table 2. The preliminary analysis results in the following partitioning of the sum of squares:

Between classes	18·2465 (19 deg. freed.)
Within classes	0·0678 (78 deg. freed.)
Total	18·3143

An ordinary analysis of variance is now conducted on the means of Table 2, the results of which are entered in Table 10.

In order to test the significance of the several items of Table 10, an appropriate error variance is calculated by dividing the intraclass variance, 0·00087, by the harmonic mean of the class numbers, 4·3548, a process which yields the number 0·00020. The analysis of variance then reduces to the form given in Table 11.

It should be noted that the appropriate figure upon which to base the errors of the class means is the intraclass variance 0·00087.

TABLE 11.

Analysis of Variance.

Variation due to	Deg. freed.	Sum. sq.	Mean sq.
Harvest	4	3·28052	0·82013
Treatment	3	0·49413	0·16471
Interaction	12	0·12673	0·01056
Intraclass	78	—	0·00020

TABLE 12.

Class Totals for Total Plant, Experiment 2.

	Treatment			
Harvest	I	II	III	Total.
2	11·0672 (6)	10·9108 (6)	10·5274 (6)	32·5054 (18)
3	16·8029 (6)	16·8283 (6)	16·4694 (6)	50·1006 (18)
4	19·8320 (6)	19·9885 (6)	19·8528 (6)	59·6733 (18)
5	19·3667 (5)	20·0322 (5)	19·7881 (5)	59·1870 (15)
6	20·0593 (5)	20·9849 (5)	21·3449 (5)	62·3891 (15)
7	20·1146 (5)	21·4889 (5)	21·7160 (5)	63·3195 (15)
Total	107·2427 (33)	110·2336 (33)	109·6986 (33)	327·1749 (99)

Figures in brackets indicate the number of observations contributing to the corresponding total.

TABLE 13.

Analysis of Variance.

Variation due to	Deg. freed.	Sum. sq.	Mean sq.
Harvest	5	75·0537	15·0107
Treatment	2	0·1542	0·0771
Interaction	10	0·4089	0·0409
Within classes	81	0·3781	0·0047
Total	98	75·9949	

(3) *Proportionate Class Numbers.*

In the data of Experiment 2 (Sudan grass), class numbers which were unequal possessed, however, the property of being proportionate, i.e.

$$n_{pt}/n_{pr} = n_{qt}/n_{qr}.$$

Under these conditions, component sums of squares of any complex item in the table of analysis of variance, such as that between classes, are additive, and conse-

quently the sums of squares corresponding to these items may be evaluated by the method given by Fisher (1934) for a single classification with unequal numbers in the classes. The following analysis of the observations on total plant, Experiment 2 (Table 12), illustrates the method.

The total sum of squares is 75·9949, and the fractions, between and within classes, are respectively 75·6168 and 0·3781. The correction factor is $327 \cdot 1749^2/99 = 1081 \cdot 2466$.

Treatment:	$(107 \cdot 2427^2 + 110 \cdot 2336^2 + 109 \cdot 6986^2)/33 =$	1081·4008
	Deduct	1081·2466
	Treatment sum sq. =	0·1542
Harvest:	$(32 \cdot 5054^2 + 50 \cdot 1006^2 + 59 \cdot 6733^2)/18 =$	395·9763
	$(59 \cdot 1870^2 + 62 \cdot 3891^2 + 63 \cdot 3195^2)/15 =$	760·3240
	Total	1156·3003
	Deduct	1081·2466
	Harvest sum sq. =	75·0537
Interaction sum sq. $= 75 \cdot 6168 - (75 \cdot 0537 + 0 \cdot 1542) =$		0·4089

The complete analysis is given in Table 13.

Variance of R and E_W.

Provided that the errors e_1 and e_2, in W_1 and W_2, respectively, are small compared with W_1 and W_2, an approximation to the error, A, of R, is given by

$$A = e_1 \frac{\partial f}{\partial W_1} + e_2 \frac{\partial f}{\partial W_2},$$

so that the variance of R is

$$v(R) = v(W_1)\left(\frac{\partial f}{\partial W_1}\right)^2 + v(W_2)\left(\frac{\partial f}{\partial W_2}\right)^2$$

$$= \frac{1}{(t_2 - t_1)^2}\left(\frac{v(W_1)W_2^2 + v(W_2)W_1^2}{W_1^2 W_2^2}\right),$$

where $v(W_1)$ and $v(W_2)$ are the variances of W_1 and W_2.

In a similar manner the unit leaf rate, E_W, has a variance given by

$$v(E_W) = v(w_1)\left(\frac{\partial f}{\partial w_1}\right)^2 + v(w_2)\left(\frac{\partial f}{\partial w_2}\right)^2 + v(W_1)\left(\frac{\partial f}{\partial W_1}\right)^2 + v(W_2)\left(\frac{\partial f}{\partial W_2}\right)^2$$

$$= \frac{1}{(t_2 - t_1)^2(w_2 - w_1)^2}\{v(w_1)A + v(w_2)B\}$$

$$+ \frac{E_W^2}{(W_2 - W_1)^2}\{v(W_1) + v(W_2)\},$$

where $$A = \{w_1 E_W(t_2 - t_1) - (W_2 - W_1)\}^2 / w_1^2,$$

and $$B = \{w_2 E_W(t_2 - t_1) - (W_2 - W_1)\}^2 / w_2^2.$$

Owing to the fact that the variances of the quantities W_1, W_2, etc., have been based on comparatively few degrees of freedom, the sensitivity of tests of significance involving the variances given above is rather low. This is, however, unavoidable owing to the nature of the data.

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PHYSIOLOGICAL ONTOGENY IN PLANTS AND ITS RELATION TO NUTRITION

2. THE EFFECT OF PHOSPHORUS SUPPLY ON THE GROWTH OF THE PLANT AND ITS PARTS.

by

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INTRODUCTION.

In the first paper of this series Ballard and Petrie (1936) have defined the effect of nitrogen supply on the drifting size and growth rate of certain gramineous plants. In this second paper we shall consider the effect of phosphorus supply on the same quantities, and also briefly the effects of the two nutrients comparatively. The definition of the effect of phosphorus supply is based mainly on two experiments, which will be described below. Some data of the first experiment have already been presented in a previous paper on transpiration (Williams, 1935).

EXPERIMENTAL PROCEDURE.

Experiment 1.

On June 7, 1933, seeds of a pure line of Algerian oats (*Avena sativa* Linn.) were planted into earthenware pots which had been filled with 14 kg. of water-washed sand. Twelve seeds were planted in each of 126 pots, and twenty-four seeds in each of a further 6 pots. On June 16 those seedlings were removed which were outside a height range based on the approximate mean height of all the seedlings. The remaining seedlings of the 126 pots were then reduced to 5 per pot; those removed constituted a uniform sample, and were used as the first harvest of the experiment.

The pots were divided into 3 groups each receiving a different amount of $NaH_2PO_4.H_2O$. The pots of these groups will be referred to respectively as those of treatments I–III. The estimates of the amounts of the reagent added per pot were corrected by analysis, the corrected estimates being:

treatment I	0·0093	gm. P.
II	0·121	„ „
III	0·486	„ „

[1] This represents one of a series of investigations carried out at the Waite Institute and financed co-operatively by the Carnegie Corporation of New York, the Council for Scientific and Industrial Research, and the University of Adelaide.

In addition, each pot received the following nutrients in solution:

$Ca(NO_3)_2.4H_2O$	0·579 gm.
NH_4NO_3	0·754 „
KCl	1·155 „
$MgSO_4.7H_2O$	0·403 „
$FeCl_3$	0·0302 „
$MnSO_4.4H_2O$	0·0013 „

The phosphate and the first three of the above nutrients were added on June 17, and the remaining three on June 19 and 20. Distilled water was then added so as to raise the water content of the sand to 2·015 l., which represented 55 p.c. of the water-holding capacity of the sand. This value was maintained by frequent additions of distilled water, and, in order to minimize the loss by evaporation, 1·5 kg. of fine gravel was spread on the surface of each pot.

Harvests were taken on June 16, 21 and 26, July 3 and 17, August 11, September 7, October 5, and November 7 and 20. The first harvest, already referred to, consisted of 6 replicates of 58 seedlings. The second harvest was made from the 6 more closely planted pots which provided 6 replicates of 4 plants per treatment. The third and fourth harvests were made by reducing the number of the plants in the 126 pots to 3 evenly-spaced plants; in each case 6 replicates of 6 plants per treatment were obtained. Harvests 5–10 were made by removing the plants from 6 pots of each treatment, each pot constituting a replicate of 3 plants. This system of harvesting was necessitated by the limited number of pots available for the experiment.

Various plant parts were separated in the manner described in the first paper of the series. The inflorescences were separated below the lowest branch of the panicle, and the consecutive harvests for which the plant parts were obtained are shown in Table 2. Tiller, leaf and panicle counts were made at the times indicated in Table 1.

All fractions except the roots were weighed as soon as possible after cutting, and were dried at approximately 98°C. overnight, and then at 85°C. until constant weight was attained. Estimations of the residual sand content were made on the dried root material and the dry weights corrected accordingly.

Between the eighth and ninth harvests a daily record was kept of the exsertion and flowering of the panicles of the primary shoots. The criterion for exsertion was the separation of the auricles of the last leaf by the inflorescence, and that for flowering was the exsertion of anthers from one or more spikelets. From this record was obtained for each treatment the date for the $\frac{1}{2}n$th plant, n being the number of plants still unharvested at that date. These dates are indicated in the graphs.

Experiment 2.

On July 26, 1934, seeds of the same pure line of Algerian oats were planted in the same pots and with the same conditions of water supply. In addition 9 seed pans were planted with 50 seeds each; these were used for the first harvest, which was taken before the application of the nutrient solutions.

As before, the pots were divided into three treatment groups that received amounts of $NaH_2PO_4.H_2O$ containing the following weights of phosphorus:

treatment I	0·0080 gm. P.
II	0·090 „ „
III	0·60 „ „

In addition, each pot received the following nutrients in solution:

$Ca(NO_3)_2.4H_2O$	1·00 gm.
NH_4NO_3	0·76 „
KCl	1·15 „
$MgSO_4.7H_2O$	0·40 „
$FeCl_3$	0·03 „
$MnSO_4.4H_2O$	0·0013 „

The phosphate and the first three of the above nutrients were added on August 7, and the remaining three on August 8.

Harvests were taken on August 6, 13 and 24, September 10, October 16, and November 16, and the final harvest in each treatment was made when translocation to the developing grain had ceased; the shrivelling of the receptacles of the spikelets was taken as an index of this stage of maturity. The dates for this final harvest were for treatment I December 19, for treatment II December 11, and for treatment III December 13. The numbers of plants per replicate for harvests 1–4 were 50, 12, 7 and 4 respectively, and for harvests 5–7 there were 3 plants per replicate. Extra pots were included in the early harvests so as to provide sufficient leaf material for analytical purposes. One pot of harvest 6 treatment II was rejected because of accidental mechanical injury to the plants.

Leaves, stems, roots, and inflorescences of the whole plant were separated for the harvests indicated in Table 3, but fresh weights were determined only for the leaf material. The inflorescence and flowering data were obtained as in Experiment 1.

The second experiment differed from the first in being planted seven weeks later in the year. The range of phosphorus supply was also wider than previously. In the first experiment the final dry weights of plants receiving treatments II and III are very similar and suggest that the nitrogen supply might have limited growth. On this account the phosphorus supply of treatment II in Experiment 2 was lowered by 25 per cent., and more calcium nitrate was applied so that the total nitrogen supply was increased by 15 per cent.

The statistical treatment of the data and the calculation of derived quantities was carried out as in the first paper (Ballard and Petrie, 1936).

PRESENTATION OF RESULTS AND DISCUSSION.

The Growth Curves.

Effects of Treatment.

The dry weight curves of the whole plants in Experiment 1 are presented in fig. 1, and those of their parts in figs. 2 and 3. The numerical data for both experiments are embodied in Tables 2 and 3. In the following paragraphs the conclusions derived from the statistical examination of the data are summarized. I–III signify plants of the three treatments, and the arabic figures refer to the harvests.

Experiment 1.

First leaf. 2, 3 and 4 taken together: I significantly below II and III.

Second leaf.[2] 3–4: I significantly below II and III; 4: III significantly below II.

First and second leaves together.[2] 5: I and III both significantly below II.

Third leaves. 5–6: I significantly below II and III; 5, 6 and 7 taken together: III significantly below II.

Total leaves. 2–10: I significantly below II and III; 5: III significantly below II, otherwise II and III are insignificantly different.

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[2] Based on t tests between individual means; all other comparisons from analyses of variance.

Roots. 5: I and III significantly below II; 6–10: I significantly below II and III.

Stems. 3–10: I significantly below II and III; 4–5: III significantly below II.

Inflorescences. 9–10: I significantly below II and III; II and III insignificantly different.

Total plant. 5–10: I significantly below II and III; 5–6: III significantly below II, otherwise II and III are insignificantly different.

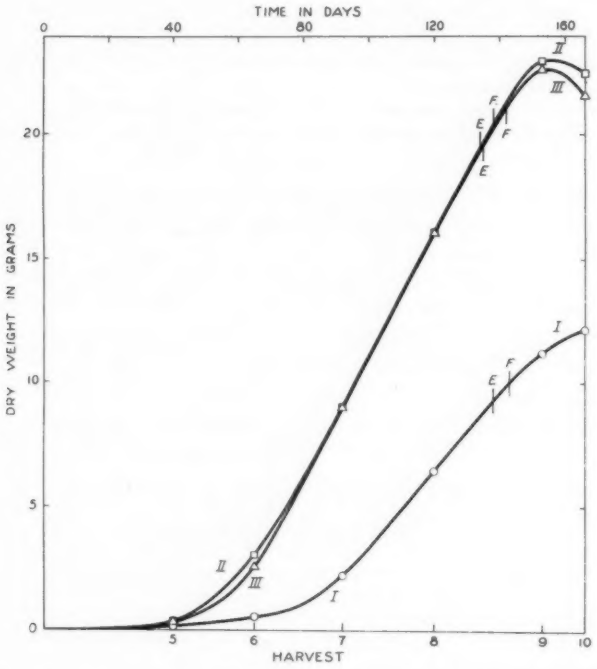

Fig. 1. Growth curves of oats with three phosphorus treatments, I, II and III, Experiment 1. In this and subsequent figures E indicates time of exsertion of inflorescences, and F time of flowering.

Experiment 2.

Leaves. 2: I significantly below III; 3–7: I significantly below II and III; 3 and 4 together: III insignificantly below II; 5, 6 and 7 together: III significantly greater than II.

Roots. 3: III significantly below I and II; 4: III significantly below II; 4–7: I significantly below II and III; 5: II significantly below III.

Stems. 2: I significantly below III; 3–7: I significantly below II and III; II and III are nowhere significantly different.

Inflorescences. 6 and 7 together: I significantly below II and III.

Total plant. 3–7: I significantly below II and III; 3: III significantly below II.

Treatment I has caused the plants to be comparatively smaller at almost all growth stages, the effect being most pronounced at harvest 6 in the case of Experiment 1, and at harvest 5 in that of Experiment 2. All the plant parts separated are also lower in weight with treatment I. Examination of fig. 6 of a previous paper

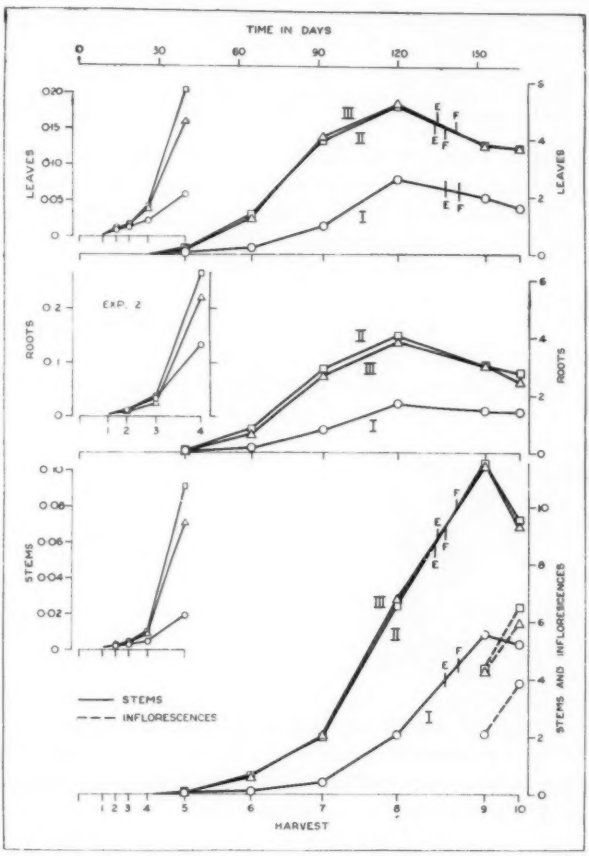

Fig. 2. Growth curves (dry weight in grams) of plant parts in oats. With the exception of the inset for roots (Experiment 2), all the curves are from the data of Experiment 1.

(Williams, 1935) shows that increased phosphorus supply has accelerated the attainment of a given proportion of the final dry weight. This was also found for total shoots by Burgevin and Guyon (1933).

During adolescence the dry weights of the whole plant and certain of its parts, especially the roots, are significantly less with treatment III than with treatment

II. The reduction is not shown by the first leaf, possibly because this organ was almost mature when the nutrients were applied. It is also less marked in Experiment 2, where the reductions in the weights of the leaves and stems are insignificant. This difference between the two experiments, and also the difference in the effect on the final dry weight with treatment II relative to that with treatment I, are almost certainly attributable to the slight differences in the nutrients supplied.

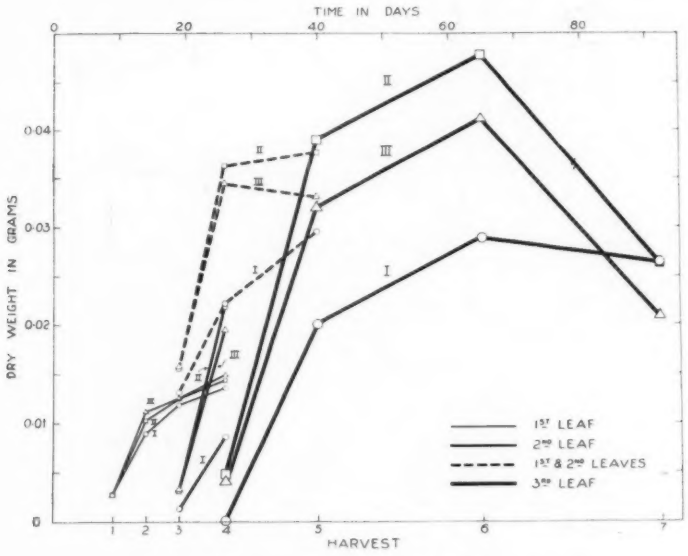

Fig. 3. Growth curves of early leaves in oats, Experiment 1.

In Experiment 2 the leaves and roots of plants of treatment III subsequently attain dry weights significantly greater than those of plants of treatment II, but these differences are not sufficient to produce significant differences in the weights of the whole plants at any of the late harvests.

Drifts with Time.

The data for the whole plant in Experiment 1 (fig. 1) suggest that dry weight maxima are attained earlier with treatments II and III than with treatment I; the changes in weight between harvests 9 and 10, however, are insignificant with all treatments. In Experiment 2, the dry weight increments between harvests 6 and 7 are individually insignificant with treatments II and III, but the increment with treatment I is significant, suggesting that treatment I has delayed senescence.

In Experiment 1 leaves and roots show dry weight maxima at harvest 8 followed by significant losses, except in the roots with treatment I. The stems show maxima at harvest 9, and these are followed by significant losses only with treatments II and III.

In Experiment 2 leaves and roots show maxima at harvest 5 with treatments II and III, and at harvest 6 with treatment I. These are followed by significant losses in weight in all cases except that of the roots with treatment I. Stems show maxima at harvest 6 with treatments II and III, and these are followed by significant losses in weight. The increase between harvests 6 and 7 with treatment I is not significant.

The dry weight maxima for the third leaves (fig. 3) all occur at harvest 6, but the subsequent loss in weight by these leaves with treatment I is insignificant, whereas the losses with treatments II and III are highly significant. At harvest 7 these leaves were brown and dry with treatments II and III, but those with treatment I were still green and turgid. The leaf counts made in Experiment 1 at harvest 8 (Table 1) show that the proportion of dead leaves per plant was greater with treatments II and III than with treatment I.

Although the incidence of maxima and the possibility of demonstrating whether gains or losses in dry weight are significant are partly determined by the time of harvest, it is evident that the above facts suggest a delay in the morphological development of the plants of treatment I. This suggestion is supported by the data for times of exsertion of inflorescences, flowering, and maturity of grain (fig. 4). In each case these phenomena occur several days later in the plants of treatment I than in the others. A slight delay is also indicated for plants of treatment III as compared with those of II, and may be associated with the initial depression noted for the plants of the former treatment.

It is possible that the initial dry weight depression with treatment II prevented the full potentiality of the high phosphorus supply being manifested within the growing period. This is rendered unlikely by the extreme similarity of the growth curves of the whole plant and all its parts with treatments II and III during later stages. Chemical analyses on the dry material of the experiments, some of which have been reported in an earlier paper (Williams, 1935), confirm this conclusion and suggest that the nitrogen supply was a limiting factor in both cases.

Relative Growth Rate.

Relative growth rates for the two experiments are presented in Tables 4 and 5 and in fig. 4. The following conclusions are drawn from the statistical examination of the data:

Experiment 1.

5–6: I significantly less than II and III; 7–8 and 8–9: I significantly greater than II and III; II and III are insignificantly different throughout.

Experiment 2.

2–3, 3–4 and 4–5: I significantly less than II and III; 2–3: III significantly less than II; 5–6: I significantly greater than II and III.

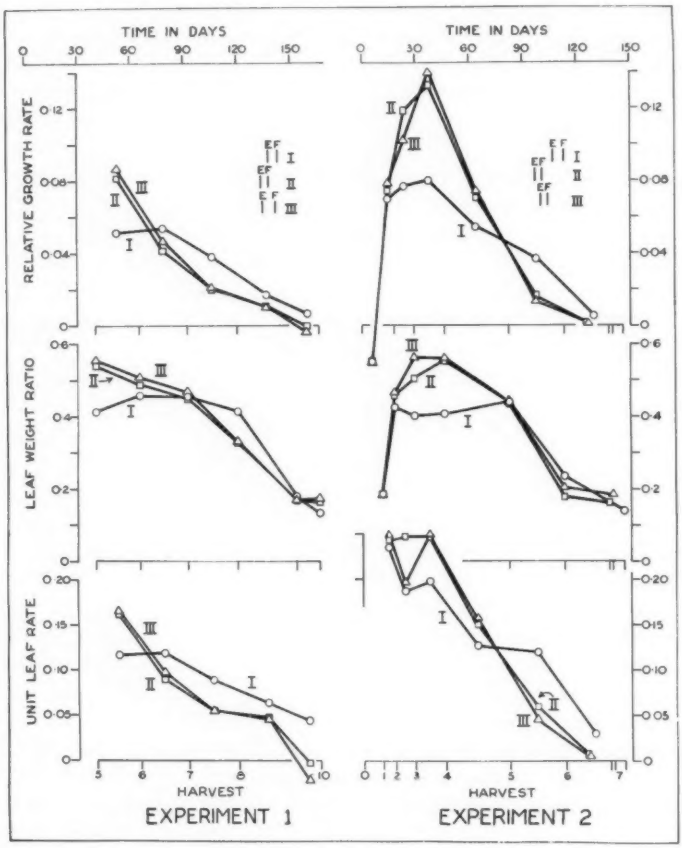

Fig. 4. Curves of relative growth rate (gm. per gm. per day), leaf weight ratio and unit leaf rate (gm. per gm. per day).

The absence of root weight data for the early harvests of Experiment 1 renders the data for R incomplete, but for the later stages of growth the two experiments present essentially the same picture. The initial negative value in Experiment 2 is attributable to the fact that between planting and the first harvest assimilation was small, and the seedlings lost weight by respiration. The treatment I curve is

below the treatment II curve for the first half of the growing period; it then crosses over and remains above. A depression of R with treatment III below that with treatment II is demonstrated only in Experiment 2 for one early harvest interval.

Leaf Weight Ratio.

The leaf weight ratio data are presented in Tables 4 and 5 and in fig. 4. The results of th statistical analyses are as follows:

Experiment 1.

5, 6 and 10: I significantly below II and III; 6: II significantly below III; 8: I significantly above II and III.

Experiment 2.

2 and 7: I significantly below III; 3 and 4: I significantly below II and III; 3: II significantly below III; 6: I significantly above II and III.

In early adolescence F_W is an increasing function of phosphorus supply. Later the values for plants of treatments II and III become insignificantly different and fall for the remainder of the life cycle. With treatment I, however, F_W remains depressed for approximately half the growing period; its curve then crosses the others, but falls to a final value which once again is lower.

Examination of the data of Tidmore (1930) for 76-day-old corn and sorghum plants shows that the leaf weight ratio decreased, and the stem weight ratio greatly increased, with increasing phosphorus supply. His data for tomatoes show first an increase and then a decrease in F_W with the same range of treatments. In attempting to interpret these and similar data, it should be remembered that the magnitude and direction of the effects on F_W and other ratios produced by varying nutrient supplies, will differ in different species, since the comparative potentiality for growth of different plant parts may be specific.

Unit Leaf Rate.

As with those of the first paper of this series (Ballard and Petrie, 1936), the unit leaf rate values are subject to an error due to the exclusion of the leaf-sheaths and flowering glumes, which perform a certain amount of carbon assimilation. The error is probably negligible during adolescence and quite small in the later stages of the life-cycle. The data are presented in Tables 4 and 5 and in fig. 4. From the statistical analyses the following conclusions may be drawn:

Experiment 1.

5–6: I significantly less than III; 7–8: I significantly greater than II and III; elsewhere the differences between I and the others just fail to be significant; II and III are nowhere significantly different.

Experiment 2.

2–3: I and III significantly less than II; 3–4: I significantly less than II; 5–6: I significantly greater than II and III.

Thus in early adolescence E_W is depressed with treatments I and III. With the latter treatment the depression passes off almost immediately, whereas with the former it continues throughout adolescence. Just before the exsertion of the inflorescences, E_W with treatment I becomes significantly greater than with treatments II and III and, although the variability of the data makes it impossible to demonstrate significant treatment differences for the later harvest intervals, the inter-treatment relations of the quantity do not alter.

Little can be said here as to the underlying causes of the observed effects of treatment on E_W; but it is possible that the early depression with treatment III is a toxic effect which influences the rate of carbon assimilation. The delayed senescence of the plants of treatment I already indicated would contribute to the high values of E_W with this treatment in the later stages of growth; but it is possible that at any given growth stage the rate of carbon assimilation of individual leaves is also higher. Gregory and Richards (1929) showed that phosphorus deficiency increased the rate of carbon assimilation of mature leaves at both low and high light intensity. The increases, however, were not significant, and moreover the experiments were conducted with high carbon dioxide concentration.

F_W and E_W as Determinants of R.

Since R is the product of F_W and E_W, an examination of fig. 4, in conjunction with the results of the statistical analyses of the variables, will reveal in what way each of the components is responsible for inter-treatment differences in R at various stages of the life cycle. For this purpose values for plants of treatment II will be used as standards of reference.

The adolescent depression of R with treatment I is contributed to by both F_W and E_W. The early depression with treatment III, however, is attributable solely to the depression in E_W at that time; the effect of the increase in F_W at about the same time tends to counteract the effect of E_W, but does not produce a significant increase in R of the plants of treatment III. The later ascendancy of the curve of R with treatment I is chiefly due to the high E_W values with this treatment. Just before and at the time of flowering F_W definitely accentuates the ascendancy, but in senescence its action is in the reverse direction.

Root, Stem and Inflorescence Weight Ratios.

Although the leaf weight ratio is of primary interest in the present study, it was thought that an examination of treatment effects on root, stem and inflorescence weight ratios would be of value. The data for these ratios are presented in Tables 6 and 7 and fig. 5. The results of the statistical analyses, performed on the inflorescence weight ratios of Experiment 1 and on all the ratios of Experiment 2, are summarized in the following paragraphs.

Experiment 1.

Inflorescence weight ratio. 10: I significantly greater than II and III.

Experiment 2.

Root weight ratio. 2–7: I significantly greater than II and III; 3–4: II significantly greater than III; 5: II significantly less than III.

Stem weight ratio. 2: I significantly less than III; 3–7: I significantly less than II and III; 4: II significantly less than III; 5: II significantly greater than III.

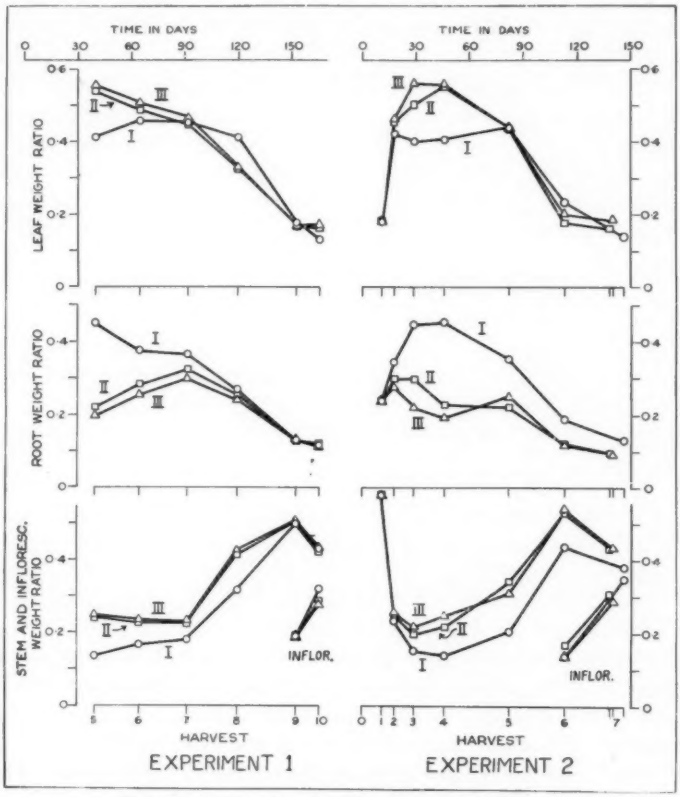

Fig. 5. Curves of ratios of parts to whole plant.

Both experiments show a very marked reduction in the root weight ratio with treatment II, and in Experiment 2 there is a further significant decrease in this ratio in early adolescence with treatment III. At harvest 5, however, there is an inexplicable reversal of the relation of the ratios of the plants of treatments II and III. At every harvest in Experiment 2 the plants of treatment I have the lowest stem weight ratios, and the inflorescence weight ratio of the plants of the same treatment are the highest at the final harvest of Experiment 1.

Fig. 6 illustrates the drifting proportions of the plant parts with treatments I and II in Experiment 2.

Other investigators provide data on the effect of phosphorus supply on ratios of plant parts to the whole plant or to the total shoots. Thus Sommer (1936) shows that, in wheat and other plants grown in solutions of large volume, root-shoot ratios are markedly decreased with phosphorus supply, especially during early growth stages. Root weight ratios derived from the data of Brenchley and Jackson (1921) show that, at a low level of nitrogen supply, an increase in phosphorus supply tends to decrease the ratio in the early stages of growth: at a higher

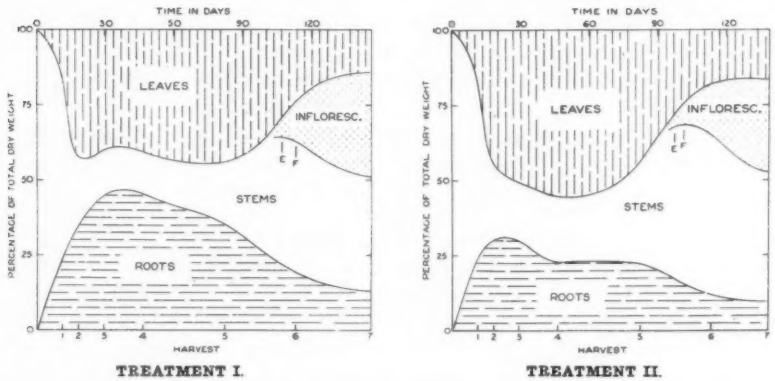

Fig. 6. Diagrams showing the relative proportions of plant parts throughout the life cycle with treatments I and II, Experiment 2. The ungerminated seed has been arbitrarily classed as stem, and the conventions of separation are those described in the first paper of the series.

nitrogen level the decrease is more pronounced and extends to later stages. The data of Parker (1927) and Tidmore (1930) all show pronounced decreases in root weight ratios with increased phosphate concentration for various plants at immature stages. The latter worker's figures demonstrate variable effects on leaf and stem weight ratios, but more would have to be known of the experimental conditions before concluding that they could not be interpreted in general terms. Data of Arrhenius (1931) with oats at the end of the life cycle show that, at low levels of nitrogen, root and grain weight ratios are independent of phosphorus supply: at higher levels the root weight ratios are decreased and the grain weight ratios increased with increasing phosphorus supply. The data of Turner (1929) are difficult to reconcile with those of other workers. He demonstrates with barley, wheat and cotton at relatively early stages of growth an increase in the root-shoot ratio with increased phosphorus supply. In pure culture experiments with excised roots of corn, he shows that increased phosphorus concentration in the medium

depresses both growth in length and the multiplication of lateral roots. To explain his other data, Turner assumes that this tendency is counter-balanced by the favourable effect of the increased phosphorus on assimilation and hence upon the supply of carbohydrates to the roots. Garola (1934) and Burgevin and Guyon (1933) show increases in grain weight ratio with increased phosphorus supply, and Mitscherlich et al. (1935) demonstrate the same effect on the grain-straw ratio except at very low levels of nitrogen; with excessive amounts of phosphorus, however, the ratio rapidly falls to zero.

The bulk of the available data thus indicates a depression in the root weight ratio with increasing phosphorus supply. Since the other ratios are affected in a more variable manner, this depression cannot be attributed to a positive stimulation of the growth of leaves or stems alone. The effect might be thought to be an osmotic one, but if this were so one would expect other nutrients to produce the same effect. Increased nitrogen does lower the root weight ratio, as is shown by Rippel and Meyer (1933) and Ballard and Petrie (1936), but the former workers also show that applications of 0, 2·0 and 10·0 gm. of potassium sulphate, at various nitrogen levels, have no significant effects 28 days after planting on the root weight ratios of their experimental plants.

The high inflorescence weight ratio of the plants receiving the lowest phosphorus supply in the present experiments is possibly associated with the delayed senescence and high final values for unit leaf rate with this treatment.

Comparison of the Effects of Nitrogen and Phosphorus Supply.

In this section an attempt will be made to compare the effects of increasing supplies of phosphorus and of nitrogen on the growth and various other attributes of the developing plant. A comparison of the growth curves in figs. 1 and 2 of the first paper of this series (Ballard and Petrie, 1936) with fig. 1 of the present paper reveals an apparent difference in the adolescent depression produced by increases in the nutrients. The 53-day dry weight curve of fig. 7 in a paper by Meyer (1927) clearly shows that the treatment relations, at this early growth stage with increasing nitrogen supply, depend on the magnitudes of the chosen supplies. Thus with 4, 6 and 8 gm. supplies of ammonium sulphate the treatment relations are as in the first paper of this series, but with 0·25, 2 and 6 gm. supplies the relations are similar to those obtained with increasing phosphorus supplies in the present paper. There is therefore no evidence that the observed differences in the adolescent depressions produced by nitrogen and phosphorus are specific responses to the nutrients.

Another obvious difference in the growth curves is to be found in the treatment relations at the later harvests. This difference is readily understood if it is assumed that nitrogen or some other essential element is limiting in the case of the two high phosphorus treatments of the present experiments. The final dry weight data of Mitscherlich et al. (1935) for oats with eleven phosphorus treatments at

ten levels of nitrogen supply are plotted in fig. 7. When allowance is made for the arbitrary choice of scales, it will readily be seen that there are no specific differences in the type of response of the plant to nitrogen and phosphorus supply with respect to the ultimate amount of dry matter produced. In particular, it should be noted that limitation in final dry weight with increasing nitrogen or phosphorus occurs at lower and lower levels as the supply of the other nutrient becomes smaller.

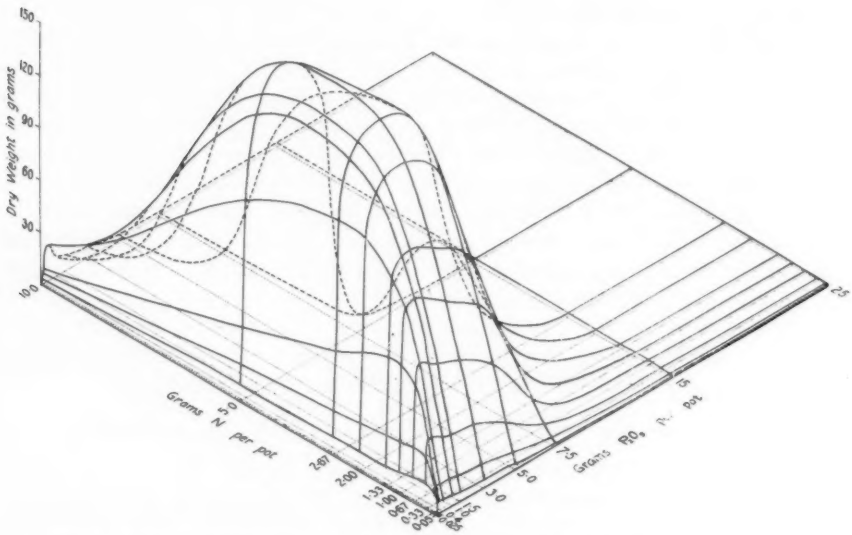

Fig. 7. Relation of final dry weight of the shoots of oats to initial supply of nitrogen and phosphorus, plotted in isometric projection. (From data of Mitscherlich et al., 1935.)

In the first paper of this series it was found that increasing nitrogen supply delayed the senescence of individual plant parts and also of the whole plant, whereas in the present experiments increased phosphorus supply hastened senescence. Good examples are provided by the growth curves of the third leaves in fig. 5 of the previous paper and fig. 3 of the present paper. These facts are easily explained by assuming that in both cases senescence is hastened by limiting nitrogen supply. Sommer (1936), with various plants grown in water culture, shows that those with low phosphate concentrations matured earlier. As all other nutrients were maintained at a constant and adequate concentration, it would seem that limiting supplies of nitrogen and phosphorus both tend to hasten senescence.

Turning now to the derived quantities specially studied in this series of papers, a comparison of figs. 8 and 9 of the first paper with fig. 4 of the present paper reveals striking differences in the effects of increasing supplies of the two

nutrients. F_W is depressed during adolescence by low supplies of both nitrogen and phosphorus; but, whereas this depression tends to continue almost to the end of the life cycle with low nitrogen, the treatment relations of F_W become reversed in the present experiments. The reversal is possibly related to the suggested limitation by nitrogen of the plants receiving the two higher supplies of phosphorus; but, since the nitrogen and phosphorus experiments were conducted with different plant species, the differences in the responses of F_W may be in part specific. During adolescence increasing nitrogen supply produced no significant effect on E_W (previous paper, fig. 9), whereas both low and high supplies of phosphorus depressed E_W for at least a part of this period (fig. 4, Experiment 2). In the wheat experiment, with a wider range of nitrogen treatment, E_W is depressed with low nitrogen supply, but unfortunately the data do not commence until late in adolescence. Thus it cannot be determined whether increasing nitrogen and phosphorus supplies have the same influence on E_W during adolescence. The observed differences in the effects on E_W, during later stages of growth, are probably associated with the stage of senescence of the leaves as a whole, and thus may not be interpreted as specific effects of the elements considered.

SUMMARY.

This paper represents the second of a series devoted to the study of physiological ontogeny, and defines the effect of different initial supplies of phosphorus on oat plants grown in sand culture.

The growth of the whole plant and its parts is limited by the lowest supply, but, apart from an initial depression with the highest supply, the other two treatments produce almost identical results. It is suggested that the plants with the latter treatments are limited by the supply of nitrogen. The onset of senescence is delayed by low phosphorus supply. For the first half of the life cycle the relative growth rate, unit leaf rate, and leaf weight ratio are depressed with low phosphorus supply. Later the values of these quantities with this treatment become the highest, but in senescence they tend to converge on those with the other treatments. In early adolescence the relative growth rate and unit leaf rate are slightly depressed, and the leaf weight ratio is increased, with the highest phosphorus supply. For the rest of the life cycle the values of all three quantities are insignificantly different with the two highest phosphorus supplies. The effect of treatment on the root, stem and inflorescence weight ratios is discussed, and finally a brief comparison is made of the effects of increasing nitrogen and phosphorus supplies on the growth and other attributes of the developing plant.

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TABLE 1.

Mean Number of Tillers, Leaves and Panicles per Plant for Experiment 1.

Date	Harvest		Treatment I	Treatment II	Treatment III
Tillers (including primary shoot)					
July 3rd	4		1	>1	>1
July 12th	4–5		1·6	3·3	3·1
Aug. 10th	6		3·9	11·6	10·5
Sept. 7th	7		6·1	13·0	13·9
Oct. 5th	8		6·9	12·7	13·2
Nov. 7th	9		5·8	13·2	13·8
Nov. 20th	10		5·8	12·3	11·8
Leaves					
July 12th	4–5		3·7	8·2	7·7
Oct. 5th	8	Living	33·0	48·3	49·3
		Dead	13·0	42·0	49·0
		Dead	28%	47%	50%
Leaves (primary shoot)					
July 12th	4–5		3·2	4·2	4·1
Oct. 5th	8	Living	6·0	6·3	6·0
		Dead	5·3	6·7	6·3
		Dead	47%	52%	51%
Panicles					
Nov. 7th	9		2·6	5·1	5·0
Nov. 20th	10		2·7	4·9	4·9

TABLE 2.

Dry Weights in gm. per Plant for Experiment 1.

Harvest	Treatment I	Treatment II	Treatment III
First Leaf			
1	0·00269 ± 0·000024	0·00269 ± 0·000024	0·00269 ± 0·000024
2	0·00897	0·01028	0·01119
3	0·0118	0·0125	0·0125
4	0·0136	0·0144	0·0149
Means of 2–4	0·0115	0·0124	0·0129

Analysis of variance for harvests 2–4 performed on unchanged dry weight data.
Interaction insignificant but treatment and time significant.
S.E. for means of 18 = ± 0·00022

Second Leaf			
3	0·00129 ± 0·000131	0·00318 ± 0·000233	0·00320 ± 0·000299
4	0·00862 ± 0·000642	0·0219 ± 0·00057	0·0195 ± 0·00068
First and Second Leaves			
5	0·0296 ± 0·00139	0·0377 ± 0·00126	0·0331 ± 0·00079
Third Leaf			
4	0·00018 ± 0·000094	0·00487 ± 0·000301	0·00403 ± 0·000417
5	0·0201	0·0389	0·0320
6	0·0289	0·0477	0·0412
7	0·0264	0·0264	0·0209
Means of 5–7	0·0251	0·0377	0·0314

Analysis of variance for harvests 5–7 performed on unchanged dry weight data.
S.E. for means of 6 = ± 0·00286
S.E. for means of 18 = ± 0·00165

Leaves of the Primary Shoot			
5	0·0536	0·1302	0·1084
6	0·0138	0·404	0·366
7	0·348	0·809	0·798
8	0·860	1·091	1·149
9	0·813	0·864	0·844
10	0·630	0·855	0·859

Harvests 1–4 as for total leaves.

Total Leaves						
1	0·00269 ± 0·000024		0·00269 ± 0·000024		0·00269 ± 0·000024	
2	0·00897	*0·952*	0·01028	*1·010*	0·01119	*1·048*
3	0·0131	*1·117*	0·0156	*1·194*	0·0157	*1·195*
4	0·0224	*1·348*	0·0412	*1·614*	0·0384	*1·584*
5	0·0587	*1·768*	0·205	*2·311*	0·160	*2·201*
6	0·234	*2·369*	1·40	*3·144*	1·27	*3·103*
7	0·988	*2·994*	3·96	*3·596*	4·12	*3·614*
8	2·62	*3·416*	5·17	*3·713*	5·25	*3·720*
9	1·98	*3·294*	3·82	*3·582*	3·80	*3·580*
10	1·61	*3·205*	3·70	*3·568*	3·71	*3·568*

S.E. for means of 6 = ± *0·0167*

TABLE 2 (Contd.)

Harvest	Treatment I		II		III	
Roots						
5	0·0642	*0·806*	0·0844	*0·925*	0·0574	*0·754*
6	0·192	*1·274*	0·844	*1·899*	0·648	*1·801*
7	0·805	*1·899*	2·92	*2·453*	2·66	*2·422*
8	1·71	*2·231*	4·07	*2·609*	3·85	*2·582*
9	1·47	*2·167*	3·00	*2·473*	3·04	*2·480*
10	1·41	*2·148*	2·77	*2·440*	2·47	*2·390*
	S.E. for means of 6 = ± *0·0353*					
Stems						
1	0·00163 ± 0·000021		0·00163 ± 0·000021		0·00163 ± 0·000021	
2	0·00203	*0·300*	0·00199	*0·295*	0·00217	*0·333*
3	0·00290	*0·461*	0·00433	*0·636*	0·00411	*0·614*
4	0·00478	*0·677*	0·00985	*0·991*	0·00855	*0·929*
5	0·0192	*1·280*	0·0911	*1·959*	0·0708	*1·849*
6	0·0849	*1·918*	0·655	*2·812*	0·586	*2·767*
7	0·393	*2·593*	1·98	*3·296*	2·05	*3·311*
8	2·01	*3·299*	6·53	*3·815*	6·77	*3·821*
9	5·56	*3·742*	11·43	*4·058*	11·28	*4·052*
10	5·21	*3·714*	9·54	*3·979*	9·32	*3·969*
	S.E. for means of 6 = ± *0·0213*					
Inflorescences						
9	2·09	*0·319*	4·38	*0·641*	4·26	*0·628*
10	3·87	*0·586*	6·33	*0·801*	5·95	*0·769*
	S.E. for means of 6 = ± *0·0187*					
Primary Shoot						
1	0·00432		0·00432		0·00432	
2	0·0110		0·0123		0·0134	
3	0·0160		0·0200		0·0198	
4	0·0271		0·0510		0·0470	
5	0·0694		0·172		0·142	
6	0·178		0·556		0·498	
7	0·486		1·23		1·20	
8	1·64		2·68		2·89	
9	4·55		5·43		5·36	
10	4·91		5·50		5·27	
	Tillers appeared between harvests 4 and 5.					
Total Plant						
5	0·142	*0·151*	0·380	*0·580*	0·288	*0·457*
6	0·511	*0·699*	2·90	*1·456*	2·50	*1·396*
7	2·19	*1·337*	8·86	*1·944*	8·82	*1·945*
8	6·33	*1·800*	15·8	*2·197*	15·9	*2·200*
9	11·1	*2·043*	22·6	*2·354*	22·4	*2·349*
10	12·1	*2·081*	22·4	*2·349*	21·5	*2·331*
	S.E. for means of 6 = ± *0·0187*					

NOTES.

In this and the next table the italic figures are means of the logarithms of the original observations. Values in Tables 2, 4 and 6 are means of 6 replicates.

TABLE 3.

Dry Weights in gm. per Plant for Experiment 2.

Harvest	Treatment I		Treatment II		Treatment III	
Leaves						
1	0·0036 ± 0·00008		0·0036 ± 0·00008		0·0036 ± 0·00008	
2	0·0136	*1·132*	0·0147	*1·167*	0·0158	*1·198*
3	0·0299	*1·475*	0·0613	*1·787*	0·0583	*1·764*
4	0·117	*2·068*	0·635	*2·802*	0·624	*2·792*
5	0·893	*2·946*	6·29	*3·797*	7·02	*3·846*
6	1·50	*3·171*	4·29	*3·632*	4·87	*3·686*
7	1·06	*3·023*	4·03	*3·605*	4·43	*3·645*
	S.E. for means of 6 = ± *0·0166*					
	S.E. for means of 5 = ± *0·0182*					
	S.E. for means of 4 = ± *0·0204*					
Roots						
1	0·0047 ± 0·00029		0·0047 ± 0·00029		0·0047 ± 0·00029	
2	0·0111	*1·043*	0·0100	*0·996*	0·0095*	*0·975*
3	0·0335	*1·521*	0·0364	*1·559*	0·0231	*1·359*
4	0·133	*2·116*	0·266	*2·420*	0·219	*2·333*
5	0·721	*2·851*	3·23	*3·502*	4·04	*3·605*
6	1·21	*3·076*	2·91	*3·461*	2·88	*3·457*
7	0·99	*2·993*	2·38	*3·376*	2·32	*3·356*
	S.E. for means of 6 = ± *0·0271*					
	S.E. for means of 5 = ± *0·0297*					
	S.E. for means of 4 = ± *0·0332*					
Stems						
1	0·0114 ± 0·00018		0·0114 ± 0·00018		0·0114 ± 0·00018	
2	0·0075	*0·871*	0·0084	*0·923*	0·0087	*0·941*
3	0·0113	*1·046*	0·0240	*1·378*	0·0227	*1·352*
4	0·0400	*1·599*	0·252	*2·400*	0·273	*2·435*
5	0·421	*2·615*	4·93	*3·690*	4·97	*3·675*
6	2·82	*3·443*	12·79	*4·106*	13·10	*4·116*
7	2·91	*3·460*	10·72	*4·030*	10·66	*4·027*
	S.E. for means of 6 = ± *0·0210*					
	S.E. for means of 5 = ± *0·0230*					
	S.E. for means of 4 = ± *0·0257*					
Inflorescences						
6	0·88	*2·932*	4·09	*3·604*	3·33	*3·509*
7	2·70	*3·420*	7·78	*3·879*	7·42	*3·820*
Sum of Means		*6·352*		*7·483*		*7·329*

Interaction insignificant. Treatment significant.
S.E. for difference between I and II, and between II and III (sums) = ± *0·140*
S.E. for difference between I and III = ± *0·136*

Total Plant						
Seed	0·0246 ± 0·00048		0·0246 ± 0·00048		0·0246 ± 0·00048	
1	0·0197		0·0197		0·0197	
2	0·0321	*1·506*	0·0331	*1·519*	0·0340*	*1·531*
3	0·0747	*1·872*	0·122	*2·084*	0·104	*2·015*
4	0·290	*2·460*	1·15	*3·061*	1·12	*3·045*
5	2·04	*3·302*	14·45	*4·157*	16·02	*4·204*
6	6·40	*3·800*	24·07	*4·381*	24·17	*4·382*
7	7·66	*3·880*	24·91	*4·395*	24·84	*4·390*
	S.E. for means of 6 = ± *0·0201*					
	S.E. for means of 5 = ± *0·0220*					
	S.E. for means of 4 = ± *0·0246*					

NOTE.

Values for harvests 2–4 are means of 6, and for harvests 5–7 means of 5. Exceptions are harvest 2, treatment III*, and harvest 6, treatment II; the latter is a mean of 4 values only. This note applies also to Tables 5 and 7.

* One root wt. replicate for harvest 2 treatment III was rejected and replaced by the mean value of the remaining 5 replicates, and the intra-class degrees of freedom reduced by unity.

TABLE 4.

Relative Growth Rate, Leaf Weight Ratio, and Unit Leaf Rate in Experiment 1.

Harvest	Treatment I	II	III
Relative Growth Rate ($100R$)			
5–6	5·12 ± 0·371	8·13 ± 0·321	8·65 ± 0·259
6–7	5·38 ± 0·371	4·14 ± 0·350	4·66 ± 0·204
7–8	3·80 ± 0·203	2·06 ± 0·194	2·10 ± 0·118
8–9	1·70 ± 0·161	1·09 ± 0·067	1·06 ± 0·065
9–10	0·67 ± 0·450	− 0·04 ± 0·233	− 0·36 ± 0·237
Leaf Weight Ratio ($100F_W$)			
5	41·3	53·9	55·5
6	45·8	48·8	50·9
7	45·4	44·9	46·7
8	41·4	32·8	33·1
9	17·9	16·9	16·9
10	13·3	16·4	17·3
		S.E. = ± 0·66	
Unit Leaf Rate ($100E_W$)			
5–6	11·63 ± 1·583	16·20 ± 1·572	16·55 ± 0·992
6–7	11·84 ± 0·913	8·95 ± 0·835	9·67 ± 0·483
7–8	8·84 ± 0·513	5·44 ± 0·406	5·40 ± 0·281
8–9	6·33 ± 0·704	4·65 ± 0·325	4·46 ± 0·282
9–10	4·34 ± 2·914	− 0·25 ± 1·421	− 2·13 ± 1·370

TABLE 5.

Relative Growth Rate, Leaf Weight Ratio, and Unit Leaf Rate in Experiment 2.

Harvest	Treatment I	II	III
Relative Growth Rate ($100R$)			
Sowing–1	− 2·02	− 2·02	− 2·02
1–2	6·97	7·41	7·80
2–3	7·68 ± 0·421	11·83 ± 0·317	10·17 ± 0·457
3–4	7·98 ± 0·376	13·23 ± 0·266	13·95 ± 0·414
4–5	5·41 ± 0·274	7·02 ± 0·171	7·40 ± 0·156
5–6	3·70 ± 0·381	1·65 ± 0·194	1·33 ± 0·125
6–7	0·55 ± 0·335	0·14 ± 0·212	0·10 ± 0·290
Leaf Weight Ratio ($100F_W$)			
1	18·3	18·3	18·3
2	42·3	44·5	46·4
3	40·1	50·4	56·1
4	40·6	55·2	55·9
5	44·0	43·7	43·8
6	23·5	17·9	20·3
7	14·0	16·3	18·5
		S.E. for means of 6 = ± 0·91	
		S.E. for means of 5 = ± 1·00	
		S.E. for means of 4 = ± 1·11	
Unit Leaf Rate ($100E_W$)			
1–2	23·5	24·3	24·8
2–3	18·7 ± 1·34	24·7 ± 1·23	19·6 ± 1·47
3–4	19·8 ± 1·51	24·7 ± 1·04	24·9 ± 1·91
4–5	12·7 ± 1·41	15·0 ± 0·97	15·7 ± 0·49
5–6	12·0 ± 1·65	5·9 ± 0·66	4·5 ± 0·48
6–7	3·0 ± 1·85	0·8 ± 1·26	0·5 ± 1·54

TABLE 6.

Root, Stem and Inflorescence Weight Ratios in Experiment 1.

Harvest	Treatment I	II	III
Root Weight Ratio × 100			
5	45·2	22·1	19·9
6	37·7	28·5	25·6
7	36·6	32·5	30·0
8	27·0	25·8	24·2
9	13·3	13·2	13·5
10	11·7	12·3	11·5
Stem Weight Ratio × 100			
5	13·5	24·0	24·7
6	16·6	22·7	23·5
7	18·1	22·5	23·3
8	31·6	41·5	42·7
9	50·0	50·5	50·7
10	43·0	42·4	43·5
Inflorescence Weight Ratio × 100			
9	18·9	19·4	18·9
10	32·0	28·9	27·7

An analysis of variance showed interaction to be significant.
S.E. for means of 6 = ± 0·84

TABLE 7.

Root, Stem and Inflorescence Weight Ratios in Experiment 2.

Harvest	Treatment I	II	III
Root Weight Ratio × 100			
1	23·9	23·9	23·9
2	34·5	30·1	27·8
3	44·7	29·9	22·2
4	45·5	23·0	19·5
5	35·4	22·2	25·2
6	19·0	12·1	11·9
7	13·0	9·6	9·3
S.E. for means of 6 = ± 0·84			
S.E. for means of 5 = ± 0·92			
S.E. for means of 4 = ± 1·03			
Stem Weight Ratio × 100			
1	57·9	57·9	57·9
2	23·2	25·4	25·7
3	15·2	19·7	21·8
4	13·9	21·9	24·7
5	20·6	34·2	31·0
6	43·9	53·2	54·1
7	38·1	43·3	43·5
S.E. for means of 6 = ± 0·82			
S.E. for means of 5 = ± 0·90			
S.E. for means of 4 = ± 1·00			
Inflorescence Weight Ratio × 100			
6	13·6	16·9	13·6
7	34·9	30·9	28·7

Interaction and treatment insignificant.

FURTHER OBSERVATIONS ON THE MILK OF THE MERINO EWE

by

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INTRODUCTION.

During the lambing season of 1933 observations were made at this laboratory on the yield and composition of the milk of six Merino ewes (Peirce, 1934). These determinations were carried out three and nine weeks, respectively, after lambing. During the past season the experiment was repeated on five ewes of the same flock, but the periods which elapsed before collection were 2, 4 and 6 weeks. In addition, a sample of colostrum was obtained from each of five similar ewes and analysed.

THE EXPERIMENT.

Colostrum was expressed by hand from the udders of five Merino ewes, the ages of which lay between two and six years. In each case the sample was obtained within a few hours after parturition, and prior to suckling by the lamb. The colostrum was preserved with one drop of formalin per 100 c.c., and kept in a refrigerator at 0·5° C. until the analyses were completed.

The procedure adopted for the determinations on milk was slightly different from that previously reported by us.

Five Merino ewes, aged three to seven years, were selected for the experiment. These ewes were considered to be producing a normal amount of milk, as the growth of their lambs during the first two weeks of life (when milk was their sole food) was within 10 p.c. of the average for the flock. Throughout the period of gestation and lactation the ewes grazed in the same paddock as in the previous experiment.

Observations were made 2, 4 and 6 weeks after lambing.

At about 9 a.m. the ewes and lambs were separated and placed in small enclosures, side by side, situated in a convenient portion of the paddock. After about two hours the lambs were allowed to suckle, and were then returned to their pen.

The time was noted, and taken as the starting point of the experiment. Thence for the next 24 hours the lambs were allowed to suckle at intervals of 2¼ hours during the day-time, being weighed before and after each drink; five milkings took place in this interval. The ewes and lambs were then allowed to return to the main flock, grazing at will in the same paddock, for 24 hours, after which the above procedure was followed for a further 24 hours. We thus measured the amount of milk produced during two periods each of 24 hours, separated by a similar interval.

Samples were drawn by hand from the ewe at alternate milkings, just before the lamb was allowed to suckle. The amount taken was roughly proportional to the quantity of milk which the ewe might be expected to give at that milking. About 40 p.c. of the total amount was taken at the first milking of the final day, following the lamb's overnight fast of 14 hours. The five samples so obtained from each ewe were bulked and preserved in the same manner as the samples of colostrum.

METHODS OF ANALYSIS.

These were the same as those used in the previous work, with the exception of the determination of energy. This was calculated from the analysis by the formula given by Overman and Sanman (1926), the values calculated previously by this formula being found to differ by only 2 p.c. from those obtained directly by combustion.

The analyses for the nitrogenous constituents gave total N, casein N and lactalbumin + globulin N. In addition, globulin + casein N was also determined on the samples of colostrum, using the method given by Allen (1932). To 2 ml. of colostrum neutralized with dilute caustic soda, 50 ml. of a saturated solution of magnesium sulphate were added. The precipitate was filtered, washed with saturated $MgSO_4$, and its nitrogen content determined by the Kjeldahl method. From the figures for casein N, lactalbumin + globulin N and globulin + casein N, the percentage of each of the nitrogenous constituents was calculated.

TABLE 1.

Composition of the Colostrum of Five Merino Ewes on a Pasture at Adelaide, South Australia, 1935.

Sheep	W_6182	W_6181	W_517	W_6199	W_422	
Age	6 years	6 years	2 years	6 years	3 years	Average.
Specific gravity	1·066	1·050	1·081	1·092	1·068	1·072
	%	%	%	%	%	%
Water	67·04	71·81	71·25	60·71	72·43	68·65
Fat	9·91	10·66	2·48	7·00	4·92	6·99
Lactose	2·49	3·26	3·06	1·41	2·62	2·57
Ash	1·14	0·86	0·96	0·94	1·02	0·98
Casein (N × 6·38)	6·77	4·32	7·29	5·95	6·42	6·15
Lactalbumin	2·13	1·37	3·13	3·94	2·30	2·57
Globulin	5·63	4·41	6·18	14·47	6·59	7·46
Non-protein N	0·013	0·055	0·024	0·123	0·115	0·066
P	0·206	0·164	0·200	0·173	0·216	0·192
Ca	0·227	0·154	0·167	0·154	0·180	0·176

TABLE 2.

Yield and Composition of the Milk of Five Merino Ewes on a Pasture at Adelaide, South Australia, 1935.

Time since lambing	2 weeks.						4 weeks.						6 weeks.					
Sheep	$W_6$194	$W_6$197	$W_2$30	W_{3B}17	$W_4$14	Aver-	$W_6$194	$W_6$197	$W_2$30	W_{3B}17	$W_4$14	Aver-	$W_6$194	$W_6$197	$W_2$30	W_{3B}17	$W_4$14	Aver-
Age	6 yr.	6 yr.	7 yr.	4 yr.	3 yr.	age.	6 yr.	6 yr.	7 yr.	4 yr.	3 yr.	age.	6 yr.	6 yr.	7 yr.	4 yr.	3 yr.	age.
Daily yield (gm.)	1,110	1,205	1,250	1,320	1,075	1,190	970	1,015	875	1,075	880	960	770	945	750	860	685	800
Specific gravity	1·034	1·034	1·035	1·035	1·038	1·035	1·035	1·035	1·038	1·037	1·038	1·037	1·035	1·035	1·036	1·037	1·039	1·037
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Water	80·91	81·92	80·90	82·48	80·50	81·34	80·64	82·04	81·32	82·49	81·26	81·55	79·98	82·38	79·83	81·25	81·47	80·98
Fat	8·64	7·89	8·54	7·41	8·29	8·15	8·80	7·49	7·76	6·37	7·64	7·61	9·37	7·32	9·29	8·00	7·16	8·23
Lactose	4·43	4·68	4·60	4·75	4·78	4·65	4·54	4·97	4·72	4·84	4·86	4·79	4·42	4·99	4·44	4·83	4·86	4·71
Ash	0·90	0·82	0·89	0·91	0·92	0·89	0·92	0·82	0·96	0·92	0·91	0·90	0·91	0·83	1·00	0·95	0·92	0·92
Casein (N × 6·38)	3·41	3·27	3·15	2·98	3·97	3·36	3·90	3·41	3·69	3·37	4·06	3·69	3·82	3·15	3·83	3·29	3·91	3·60
Lactalbumin	0·77	0·74	0·76	0·74	0·65	0·73	0·87	0·82	0·82	0·61	0·59	0·74	0·68	0·51	0·67	0·71	0·72	0·66
Non-protein N	0·057	0·019	0·084	0·021	0·061	0·048	0·023	0·024	0·058	0·052	0·026	0·037	0·066	0·071	0·092	0·074	0·062	0·073
P	0·124	0·118	0·126	0·129	0·131	0·126	0·127	0·114	0·140	0·133	0·135	0·130	0·126	0·120	0·143	0·132	0·129	0·130
Ca	0·188	0·170	0·193	0·204	0·205	0·192	0·201	0·183	0·225	0·212	0·205	0·205	0·212	0·186	0·224	0·222	0·206	0·214
Energy (Cals. per gm. milk)	1·26	1·17	1·25	1·12	1·26	1·21	1·28	1·15	1·20	1·07	1·19	1·18	1·34	1·12	1·34	1·21	1·16	1·23

RESULTS.

Table 1 shows the composition and specific gravity of the colostrum of five Merino ewes.

The daily yield, specific gravity, composition and calculated energy content of the milk of five similar ewes, 2, 4 and 6 weeks after lambing are shown in Table 2. The average values from this table are combined with the averages obtained in the 1933 season to form Table 3, which shows the changes which occur as lactation advances.

DISCUSSION.

1. Colostrum.

The composition of the colostrum of the ewe as found by various workers is set out below.

Water.	Fat.	Sugar.	Casein.	Albumin.	Globulin.	Ash.	Authority.
47·03	25·04	1·54	4·96	18·56		1·19	Weiske & Kennepohl quoted by Richmond (1920)
60·71–72·43 (68·65)	2·48–10·66 (6·99)	1·41–3·26 (2·57)	4·32–7·29 (6·15)	1·37–3·94 (2·57)	4·41–14·47 (7·46)	0·86–1·14 (0·98)	Peirce (1934)
			Total Protein.				
69·74	2·75	8·85	17·35			1·29	Voelcher quoted by Richmond (1920)
	5·5–15·5		14·91–23·12				East Friesian Milk Sheep — B, (1934)
	2–20		10·48–26·01				Mutton Merino Sheep — B, (1934)

Earle's (1935) estimations of the proteins of ewe's colostrum are of interest in this connection.

Fractionation of Protein in Ewe's colostrum, drawn ½ hour before lamb first suckled (Earle, 1935) (expressed in gm. Nitrogen per 100 c.c.).

Ewe No.	Euglobulin.	Globulin. Pseudoglobulin 1 and 2.	Total.	Casein.	Albumin.	Non-protein N.	Total N.
25A	0·718	0·472	1·190	1·117	0·553	0·107	2·967
18E	1·738	0·525	2·263	1·205	0·282	0·307	4·057
23B	0·817	0·756	1·573	1·626	0·396	0·038	3·633
17G	0·980	0·730	1·710	1·556	0·540	0·256	4·062
Mean			1·684	1·376	0·443	0·177	3·680

The results show the divergence between the colostrum of different breeds of sheep. The wide range in the composition within a uniform line of sheep, inbred for many generations, is shown in Table 1, where the amount of the various constituents is seen to differ from sheep to sheep by several hundred per cent. Under these circumstances the average value has little meaning.

The principal difference between colostrum and milk was the much larger content of solids in the former, due to the increase in the nitrogenous constituents. Casein was present in almost twice and lactalbumin to more than three times the amount present in milk during the second and third weeks. The greatest increase, however, was in the globulin fraction which, from being found in milk in but a trace, was present in one sample of colostrum to the extent of over 14 p.c., averaging over 7 p.c. Fat and lactose were also very variable, the latter occurring in smaller quantity than in milk.

TABLE 3.

The Change in Yield and Composition of the Milk of the Merino Ewe with Advance in Lactation.

Year	1935.	1933.	1935.	1935.	1933.
Time since lambing	2 weeks.	3 weeks.	4 weeks.	6 weeks.	9 weeks.
Daily yield (gm.)	1,190	1,205	960	800	650
Specific gravity	1·035	1·036	1·037	1·037	1·039
	%	%	%	%	%
Water	81·34	82·34	81·55	80·98	80·71
Fat	8·15	7·41	7·61	8·23	7·90
Lactose	4·65	4·83	4·79	4·71	4·81
Ash	0·89	0·86	0·90	0·92	0·90
Casein	3·36	3·43	3·69	3·60	4·38
Lactalbumin	0·73	0·81	0·74	0·66	0·85
Non-protein N	0·048	0·051	0·037	0·073	0·046
P	0·126	0·095	0·130	0·130	0·129
Ca	0·192	0·195	0·205	0·214	0·205
Energy (Cals. per gm. milk)	1·21	1·13	1·18	1·23	1·22

2. Milk.

Large-framed Merino ewes of the Mid-North of South Australia were used for this experiment. They were of the some flock as those used in the work in 1933, and were grazed in the same paddock under similar climatic conditions. These considerations, together with a similar rate of growth of the experimental lambs in the two seasons (doubling of the birth rate in 17 days) suggest that one might regard the results of the two periods as portions of the same experiment. This has been done in Table 3, in which the yield and composition of the milk at various stages of lactation is shown.

From this table it is seen that the milk two and three weeks, respectively, after lambing was similar. The fat, always a variable constituent, showed some difference, and this change was responsible for the difference in energy content. In addition, phosphorus in 1933 was present only to the extent of 75 p.c. of its value in 1935, but no explanation for this divergence is apparent. Other than these changes the yield and composition at the two stages in lactation were almost identical.

After this time changes were noticeable, somewhat slight at 4 and 6 weeks,

but more pronounced at 9 weeks. The yield decreased quite regularly until at 9 weeks the amount secreted was but slightly more than one-half of that of the first 3 weeks. Increases of the order of 5 p.c. occurred in total solids, calcium, ash, and energy, during the same interval, while that of phosphorus was of the order of 15 p.c. Casein increased by about 7 p.c. up to 6 weeks, but in the ninth week the increase was almost 30 p.c. Lactalbumin and lactose remained constant, while fat was variable, but did not show a tendency to rise.

A comparison of the results with those of several investigators was presented with our former publication, and as the same general conclusions hold from our work now as previously, it does not appear to be necessary to repeat this phase of the work.

SUMMARY.

Colostrum collected from five Merino ewes on pasture was analysed. The average percentage composition was: Total solids, 31·35; fat, 6·99; lactose, 2·57; casein, 6·15; albumin, 2·57; globulin, 7·46; ash, 0·98; phosphorus, 0·19; and calcium, 0·18. The specific gravity was 1·072.

The daily yield of milk from five similar Merino ewes 2, 4 and 6 weeks after lambing, was measured, and the composition of the milk at these times was determined. With these results are presented those obtained two years previously with sheep of the same flock, but 3 and 9 weeks, respectively, after parturition. The changes occurring during the course of lactation were noted.

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THE CHEMICAL MEDIATION OF IMPULSES IN CERTAIN FIBRES OF THE SPLANCHNIC NERVES

by

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It has been suggested by Dale (1934) that a classification of the autonomic nervous system may be made according to the nature of the chemical mediator which has been found to be liberated at the terminations of the nerve fibres during their activity. Thus he describes the autonomic nerves as either "cholinergic" or "adrenergic". Although some parts of the sympathetic system are cholinergic, many of its distributions exhibit an activity of such a nature as to make the term adrenergic applicable to the nerves. The close correspondence that exists between the results of activity of adrenergic nerves and the effects of the injection of adrenaline has been referred to by Cannon (1934). He pointed out, however, that the more recent investigations of Cannon and Rosenblueth (1933) have led to the concept that the chemical mediator of these nerves is not adrenaline but an adrenaline-like substance which has been named "sympathin" by Cannon and Bacq (1931). Whereas adrenaline causes a fall in the blood pressure of an animal in which the vasoconstrictors have been paralysed by large doses of ergotoxin, sympathin may effect a rise. The separate existence of sympathin has not been universally accepted: Dale (1934) believes that adrenaline is likely to prove to be the chemical mediator; and Bacq (1935) states that two mediators of autonomic impulses exist—acetylcholine, which is liberated by the cholinergic nerves, and adrenaline, which is liberated by the adrenergic.

The present paper describes experiments in which stimulation of the splanchnic nerves has been done in various preparations. The results found have been examined with a view to determining what substance or substances are indicated as the chemical mediator of impulses in the splanchnic nerve fibres which are distributed elsewhere than to the adrenal gland.

EXPERIMENTAL.

The experiments were made on dogs. Premedication with morphine was adopted, and the anaesthetic used was chloroform.

The splanchnic nerves on one side, usually the left, were approached through an incision along the line of the lateral border of the erector spinae. When the nerves were secured they were divided as far proximally as possible. Artificial respiration was used in order that the operative field might be extended somewhat into the thorax. Blood pressure tracings were made from the carotid artery. The external jugular vein was prepared for intravenous injections. In all cases,

the animals were fully ergotoxinized, Burroughs Wellcome "ergotoxine enthanosulphonate" being used. The ergotoxin in solution in saline containing 1 mg. per c.c. was administered intravenously. The dogs were considered to be fully ergotoxinized when the injection of 5 c.c. of 1: 100,000 adrenaline led to a fall of blood pressure. The amount of ergotoxin used to produce this effect varied up to 6·7 mg. per kg.

Faradic stimulation of the distal end of the divided splanchnic nerves was applied for 60 seconds, the secondary coil being kept at the same distance from the primary in all the experiments.

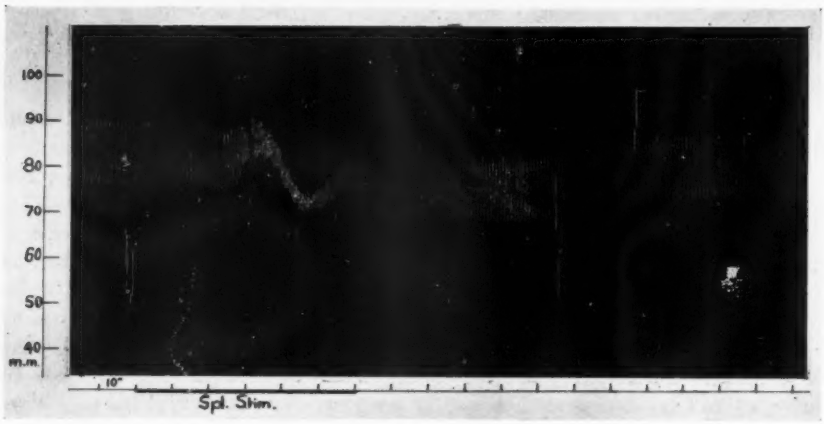

Fig. 1. Effect of splanchnic stimulation for 1 minute on the blood pressure of a dog which had received 4·5 mg. ergotoxin per kg. Time in 10 seconds.

In one series of experiments, adrenalectomy was performed on the same side as the exposed splanchnic nerves, care being taken to avoid, as far as possible, damage to the sympathetic plexuses and the coeliac ganglion. In another series of experiments, the abdominal aorta, the superior mesenteric artery, and the coeliac axis were secured through the original incision, and were clamped at the appropriate stage of the experiment. Since clamping of the abdominal aorta above the coeliac axis might include the phrenico-abdominal artery and so deprive the adrenal gland of all its sources of blood-supply, the procedure outlined was preferred in order to ensure an adequate blood-supply to the gland.

RESULTS.

In testing for the reversal of the adrenaline effect on the blood pressure by ergotoxin, it was found that the intravenous injection of adrenaline frequently caused a brief, weak pressor response which preceded the strong fall. As much as 50 p.c. more ergotoxin did not alter this pressor response which, as Dale (1913) stated, is due to the cardioaccelerator action of adrenaline becoming effective slightly before the vasodilator action.

In the first series of experiments, the effect on the blood pressure of splanchnic stimulation was first observed (Fig. 1). It is seen that the strong rise of blood

pressure which results from splanchnic stimulation in the untreated animal was replaced by a series of alternating rises and falls in the ergotoxinized. Adrenalectomy was now performed, and the splanchnic nerves were again stimulated. The effect of the absence of the adrenal gland was that the previously-obtained alternating rises and falls of the blood pressure gave place to a more sustained rise (Fig. 2). This was, however, intermittently interrupted by slight falls which were not

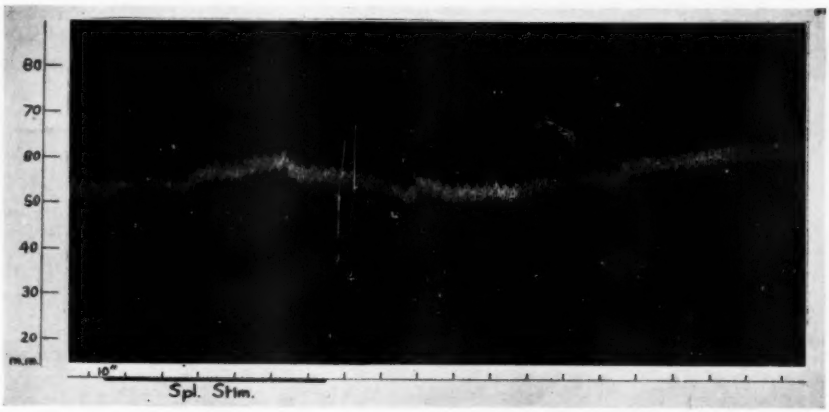

Fig. 2. Effect of splanchnic stimulation for 1 minute on the blood pressure of the dog of Fig. 1 after adrenalectomy. Time in 10 seconds.

sufficient to reduce the blood pressure below the level existing before splanchnic stimulation. This preponderance of the pressor response differentiates the blood pressure changes after adrenalectomy from those in the intact ergotoxinized animal. Similar results were found in the experiment in which the left splanchnic nerves were divided and the left adrenal removed before the tests were carried out on the right side.

In the second series of experiments the usual procedure was followed to the stage of the first stimulation of the splanchnic nerves (Fig. 3). The abdominal aorta below the superior mesenteric artery, the superior mesenteric artery and the coeliac axis were then clamped. After the blood pressure had become steady at the resulting higher level, the splanchnics were stimulated. This resulted in a fall in blood pressure (Fig. 4). Sometimes this fall was preceded by a weak, brief rise due to cardioacceleration, as was found when adrenaline was injected. Finally, after adrenalectomy had been performed, the arterial clamps being left in position, splanchnic stimulation was found to be entirely without effect on the blood pressure.

DISCUSSION.

Stimulation of the splanchnic nerves in the fully ergotoxinized dog causes alternate rises and falls in the blood pressure. In the tracings shown (Fig. 1), the blood pressure changes which were appreciably sustained were four rises and three falls. Such findings are compatible with the assumption that at least two factors or substances having, under the experimental conditions, respectively vasoconstrictor and vasodilator effects, are acting simultaneously. One of these substances is the adrenaline liberated from the adrenal gland. Adrenalectomy, therefore, would eliminate this factor. Splanchnic stimulation now (Fig. 2) results in

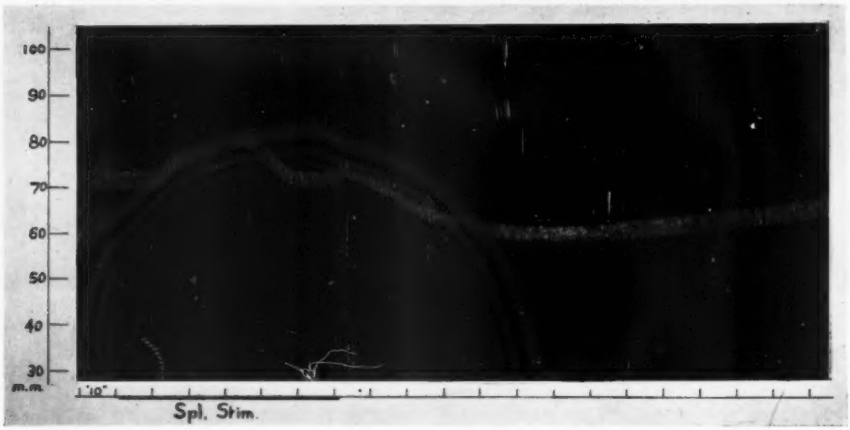

Fig. 3. Effect of splanchnic stimulation for 1 minute on the blood pressure of a dog which had received 6·7 mg. ergotoxin per kg. Time in 10 seconds.

a blood pressure which at first shows a general upward trend intermittently interrupted with slight relative falls and, later, shows a steady rise. The obvious feature of the tracing is that the removal of the adrenal gland has very greatly reduced the previously observed vasodilatation which, however, has not entirely disappeared. This persistance of vasodilatation will be found to be in agreement with the results of Cannon and his co-workers. Cannon and Uridil (1921) found that splanchnic stimulation in adrenalectomized cats led to the discharge of an adrenaline-like substance from the liver. As a result, the blood pressure rose, the animals not having been ergotoxinized. In the ergotoxinized cat, Cannon and Rosenblueth (1933) from examination of the effects of stimulating the nerves on the duodeno-hepatic artery concluded that two kinds of chemical mediator are concerned. These they named Sympathin E (excitatory) and Sympathin I (inhibitory). Sympathin E, which is produced by stimulation of the hepatic nerves,

causes a pressor response even in the ergotoxinized animal. Sympathin I, like adrenaline, causes a vasodilatation in the ergotoxinized animal. In the work here reported the nerves stimulated included fibres to the liver and to the duodenum. Therefore, even after adrenalectomy, there would be expected, as was indeed found, evidence of simultaneous pressor and dilator effects. The pressor response is the stronger, but until the effect on the blood pressure of the activity of the various distributions of the splanchnic nerves is fully known, it will not be possible to make a complete statement of which group fibres distributed elsewhere than to the adrenal gland contribute to the vasoconstrictor effect and which contribute to the vasodilator.

Fig. 4. Effect of splanchnic stimulation for 1 minute on the blood pressure of the dog of Fig. 3 after the abdominal aorta below the superior mesenteric artery, the superior mesenteric artery and the coeliac axis had been clamped. Time in 10 seconds.

The chief conclusion to be drawn from the present experiments with the adrenalectomized animal is that there are, in the portion of the splanchnic nerves which is not distributed to the adrenal gland, fibres which liberate during their activity a substance which is instrumental in evoking a pressor response. This substance, obviously differing in its effect on the blood pressure from adrenaline discharged into the circulation either from the adrenal gland or by injection, is the chemical mediator of these fibres. Its indicated action, affording confirmation of the concept of Sympathin E of Cannon and Rosenblueth, is that of contributing the pressor component to the complex response of the blood pressure of the fully ergotozinized intact animal to splanchnic stimulation.

The clear separation of the chief vasodilator component of the complex response was attempted in the second series of experiments. Here the blood-supply of all structures below the diaphragm, with the exception of the adrenal gland, was stopped by means of clamping the arteries as described above. The result of

splanchnic stimulation in this preparation is a fall in blood pressure. The course of the fall is the same as that found with adrenaline intravenously administered. Frequently, too, the weak brief rise of blood pressure due to cardioacceleration by adrenaline is found in the tracings with splanchnic stimulation. It is clear that splanchnic stimulation produces an effect which is due to the discharge of adrenaline from the adrenal gland, and that no other unrecognized factors are active in the production of the depression of the blood pressure. Final confirmation of this conclusion is afforded by splanchnic stimulation after adrenalectomy has been performed in this preparation: the blood pressure remains quite unresponsive.

SUMMARY.

The pressor response of the normal animal to splanchnic stimulation is found, by examination in the ergotoxinized dog, to be contributed to by a number of components:

(1) Adrenaline is discharged from the adrenal gland.

(2) From some visceral terminations of the splanchnic nerves elsewhere than in the adrenal, a substance is released which has a pressor action, even in the ergotoxinized dog.

(3) From other visceral terminations of the splanchnic nerves not in the adrenal is released a substance which lowers the blood pressure of an ergotoxinized dog.

ACKNOWLEDGMENT.

The authors thank Professor W. A. Osborne for his advice and encouragement during the progress of this work.

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STUDIES ON A FLUORESCENT SUBSTANCE PRESENT IN PLANTS[1]

I. PRODUCTION OF THE SUBSTANCE AS A RESULT OF VIRUS INFECTION AND SOME APPLICATIONS OF THE PHENOMENON.

by

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(Submitted for publication 15th June, 1936.)

INTRODUCTION.

While engaged on work in connection with the rate of development of primary lesions of the virus of tomato spotted wilt (T.S.W.) on the leaves of artificially inoculated plants of *Nicotiana tabacum* (var. Blue Pryor) difficulty was often experienced in deciding whether certain small sunken areas were incipient lesions or merely small pits. Although any error introduced into a count on this score would be evenly distributed over the whole set of test plants, this may not necessarily be the case when the rate of development of lesions on one set of plants is being determined. For this reason some means of distinguishing between the alternatives at the time of each count seemed to be desirable. For various reasons it seemed likely that a differential absorption of ultra-violet light might take place in and near a true lesion. Some plants on which lesions had begun to develop were therefore removed to a dark room and examined under filtered ultra-violet light, when a quite unexpected phenomenon was observed. Viewed in this way the necrotic lesions stood out as dark spots, and were surrounded at their edges by bright bluish fluorescent rings or haloes. An investigation of this phenomenon was begun, and some of the findings are recorded in this paper.

TECHNIQUE.

The source of ultra-violet light was a quartz mercury-vapour lamp[2] fitted with a glass filter which eliminated practically the whole of the visible spectrum as well as the far ultra-violet. This filter transmitted light between 3,000 Å and 4,200 Å, with a maximum transmission of 3,800 Å. Whenever the phrase "ultra-violet light" is used in this paper, it refers to the rays transmitted by this filter.

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research (Australia).

[2] The lamp was loaned by the Agronomy Department of this Institute, and is used by them for strain tests of rye grass and other pasture species.

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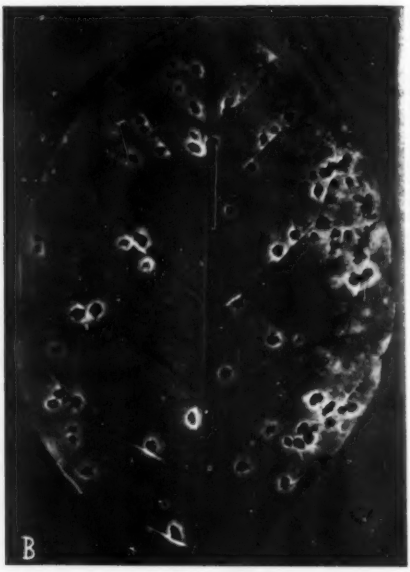
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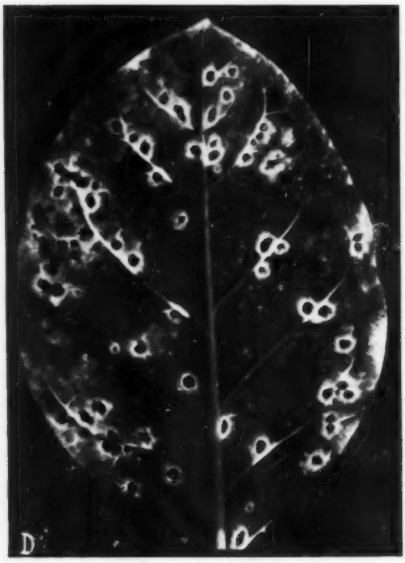
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The plants were raised in pots under glasshouse conditions, and unless otherwise stated, the plants were examined while still growing in pots after having been trimmed as for quantitative studies by the primary lesion method.

EXPERIMENTAL RESULTS.

General Description of the Phenomenon.

The *colour* of the fluorescent light is a bright blue near actively spreading lesions. The colour changes during the later stages of lesion development to a bluish-white, and in some cases there is a further change to a bright yellow.

The *position* of the fluorescence on the leaf corresponded with what appeared to the unaided eye in daylight to be normal healthy green leaf tissue immediately surrounding the necrotic lesion. These points are shown very well by the photographs[3] in Fig. 1. Figures 1A and 1C show the appearance of the upper and under surfaces of the leaf respectively when viewed in daylight. Figures 1B and 1D show the same leaf under filtered ultra-violet light. The same length of exposure was given when photographing under and upper surfaces, and the plates were developed at the same time under the same conditions, so that some index of the relative intensities of the fluorescent light is given by the photographs. It is seen that the intensity of the fluorescence is much greater on the under surface of the leaf. This difference is probably largely due to the structure of the leaf, from which we would expect a greater absorption of the incident ultra-violet light (and also of the emitted fluorescent light) to take place within the upper layers. There is also some evidence to show that the fluorescent substance undergoes a change on exposure to light, resulting in a less intense fluorescence. Since the upper surface of the leaf receives more light than the under surface, it is very likely that a photochemical action may be in part at least responsible for the observed difference. Also necrosis is always more severe and localized when the light intensity during the period of lesion development is high; and from general observations it appears that the maximum amount of fluorescent material is formed when the type of injury is intermediate between a severe localized necrosis and the comparatively slight injury caused by a systematic infection without visible necrosis. All these factors would operate to give the observed difference.

[3] The author is indebted to Mr. A. D. Cocks for his care and patience in taking the photographs reproduced in this paper.

EXPLANATION OF FIGURES.

Fig. 1. Photographs of a leaf of *Nicotiana tabacum* showing primary lesions produced by the virus of tomato spotted wilt.

A and **C.** Upper and under surfaces, respectively, photographed in daylight on a panchromatic plate.

B and **D.** Showing the fluorescent haloes surrounding the lesions. Upper and under surfaces, respectively, photographed under filtered ultra-violet light. (The incident light was cut out by interposing an aesculine filter between the leaf and the camera lens so that the leaf was photographed by the light of its own fluorescence.)

The necrotic spots, which contain a quantity of purple-brown pigment, fluoresce with a dull red colour, which in most cases is only just perceptible to the eye, and does not show up in the photographs. The dark areas under ultra-violet light correspond exactly with the necrotic areas, and this is clearly evident from a comparison of the daylight and ultra-violet photographs of Fig. 1. The bright haloes in Figs. 1B and 1D surrounding these necrotic spots correspond with the fluorescent areas. When taking photographs with ultra-violet light an aesculine filter was interposed between the leaf and the camera lens, thus cutting off the incident light. The object was therefore photographed by the light produced by its own fluorescence.

A number of small fluorescent spots and circles are visible in B and D where no corresponding spots appear in A and C. This is quite common, and incipient lesions may be picked up by viewing leaves under ultra-violet light a day or two before they become visible to the eye in daylight.

The course of lesion development as seen under ultra-violet light is briefly as follows: At first a small fluorescent disc is visible before necrosis takes place. The fluorescent disc becomes larger as it spreads radially outwards, and soon the centre of the disc loses its bright fluorescence as the tissue in this region becomes necrotic. The appearance from then on is a dark central disc (corresponding with the necrotic area) surrounded by a bright fluorescent halo. The necrotic area and the fluorescent halo both move outward radially during the course of days until several lesions meet or a larger vein is encountered, in which latter case the fluorescence travels along the vein in both directions. Under suitable conditions the fluorescence travels down a lateral vein to the midrib, and thence along the midrib to the petiole. It is interesting to note that the fluorescent rings in the mesophyll tissue correspond with the positions where the virus is actively multiplying.

General Character of the Fluorescent Substance.

The fluorescence of many plants and plant tissues under ultra-violet light has been described by numerous workers, and a good summary of the literature is given by Radley and Grant (1933). It appears that almost all parts of plants fluoresce in some way or other, so that the presence of a fluorescence in a plant is in itself in no way unusual. However, the particular fluorescent substance considered here has certain properties which characterize it and enable it to be distinguished from others present. It appears to be similar to that reported by Marx and Merkenschlager (1932) to be present in potato tubers showing degeneration (Abbau) disease.

On placing the freshly-picked diseased leaves in distilled water for a short time a strongly fluorescent infusion is obtained, and the leaf is found to have lost the greater part of its fluorescence. As is to be expected this infusion contains many things besides the fluorescent substance, and since this is present in relatively small amount (its fluorescent powers are extremely high), large numbers of plants

are required to give a workable quantity. During the past twelve months the substance has been extracted from a large number of plants, and the concentrates are now being purified. However, by virtue of its fluorescence, it has been possible to determine a number of its properties by working with only partially purified material. The following properties are listed in order to give some idea of the nature of the substance.

The substance is soluble in water, ethyl alcohol, chloroform, and many organic solvents. The colour of the fluorescent light varies with the solvent and, in aqueous solutions, with the pH value. Solutions of the substance may be boiled without apparent loss of fluorescent intensity. It is very stable to acid conditions, but is destroyed by concentrated sulphuric acid and sodium hydroxide solution. Solutions fluoresce much more brightly in alkaline solution (e.g. pH 8) than in acid solution, and the character of the fluorescent light is different. A shift to longer wave lengths takes place as we go from acid to alkaline solutions, passing from blue tinged with voilet through a whitish-blue to greenish-yellow at very high pH values. These changes are reversible except at high alkalinities, where an irreversible change takes place. Observations with a visual quartz ultra-violet spectrometer[4] show that the absorption bands undergo a similar change with changing pH value.

It is very likely that those colour changes noted in a previous section (p. 201), through which the fluorescence passes as the lesions age, are due to the changing pH value of the injured tissue. Although the red fluorescence of chlorophyll would affect the colour as seen in the leaf itself, the sequence of changes is exactly paralleled by those occasioned by changing the pH value in solutions free of other fluorescent substances.

Blotting Paper Traces of Fluorescent Leaves.

When leaves carrying primary lesions are pressed between sheets of white blotting paper in the usual way for preparing museum specimens, the leaves lose their fluorescence, but the blotting paper (which looks quite blank in daylight) shows a bright blue-violet fluorescent outline of the leaf when viewed under ultra-violet light. A photograph of such a piece of blotting paper is shown in Fig. 2. In this case a slight accidental shift of the leaf before it was completely pressed has caused an outline of the lateral veins to be superimposed on the main trace. This trace, however, shows up the outline of the lateral veins to a more marked degree than any others obtained, and is in consequence reproduced here in spite of its other faults. Similar traces have retained their bright fluorescence for over eighteen months. It will be observed that the fluorescent substance has not spread to any considerable extent in the blotting paper, and the dark centres of the lesions show up plainly. An interesting point brought out by these tracings is that often the greater part of the vascular system was outlined in a fluorescent trace on the

[4] The author is indebted to Professor Kerr Grant for the loan of this instrument.

paper as well as the neighbourhood of the lesions, although only the outer edges of the lesions themselves were visibly fluorescent when the leaves were examined before pressing. Apparently the fluorescent substance is transported from the neighbourhood of the lesions to the vascular bundles, since the fluorescence of the veins develops only after the fluorescent haloes have become established.

Fig. 2. Photograph showing the fluorescence of a piece of white blotting paper on which a leaf of *N. tabacum*, carrying primary lesions of T.S.W. virus, had been pressed. Photographed under filtered ultra-violet light with an aesculine filter between object and camera. Viewed in daylight this piece of paper was a plain white, and indistinguishable from a clean unused piece of blotting paper.

The Fluorescent Substance as a Normal Constituent of Healthy Plants.

The upper surface of normal healthy green tobacco leaves showed only the characteristic red fluorescence of chlorophyll when viewed under ultra-violet light. This red fluorescence was more pronounced on the under surface, but here the midrib and principal lateral veins often gave a bluish-white fluorescence. The fluorescent substance is usually absent from the blotting paper trace of a pressed healthy leaf except at the base of the petiole, where a faint blue fluorescence is

observed. Occasionally this extends further along the midrib, and sometimes to the lateral veins also. The amount of the substance as judged by the intensity of the fluorescence is, however, very small, and it has not been demonstrated in other parts of the leaf by this method. In making these comparisons between healthy and diseased leaves, plants from the same batch were used. These were usually about two months old. Further, examination of hand sections across the stem, petiole, midrib, and lateral veins showed that the fluorescent substance was a normal constituent of healthy plants, but was usually present in healthy plants of the same age as the diseased ones in such small amounts that the exciting ultra-violet rays had to be concentrated by a lens to show it up clearly. Viewed in this way, the substance is seen to be associated with the vascular bundles. Work on the distribution and movement of the substance in the plant is at present in progress, and will be reported later. The point of importance for the purpose of this paper is that the substance is present in relatively small quantity in healthy plants, but that it is present in relatively large quantities as the result of infection with the virus of tomato spotted wilt. That the enhanced fluorescence of diseased leaves is due to the presence of more of the substance and not to a masking of it in the healthy leaf, is shown by the blotting paper traces, and also by crushing the leaves and clarifying the expressed juice when that from the diseased leaves fluoresces brilliantly even after extensive dilution, whereas that from healthy leaves fluoresces exceedingly faintly.

Production of the Fluorescent Substance as the Result of Systematic Invasion of Tobacco Plants by T.S.W. Virus.

Systematic infection of tobacco plants by T.S.W. virus has been described by Samuel and Bald (1933). The description given below refers to artificially inoculated plants raised in the glasshouse.

When tobacco plants are inoculated with T.S.W. virus the first reaction is the multiplication of the virus in localized areas on the inoculated leaves, giving rise to primary lesions. At low temperatures the infection spreads only slowly beyond the inoculated leaves especially when the light intensity is also high. However, when the prevailing temperatures are moderate or high, the virus spreads more extensively to parts of the plant other than those inoculated. The symptoms of this systematic infection vary with the conditions of temperature and light prevailing at the time. Normally, however, the presence of virus is first made manifest by lines of necrotic tissue bordering the midrib and lateral veins. The necrosis spreads across the mesophyll tissue between lateral veins, eventually causing the collapse of the leaf. An early stage of this systemic invasion is shown in Figs. 3 and 4. As seen in the photographs by daylight (A and C) the necrosis is not extensive. The photographs taken under ultra-violet light (B) show the dark areas corresponding with the necrotic tissue, and a brilliant fluorescence extends into the vascular network, and is seen to be much more extensive than the necrotic area.

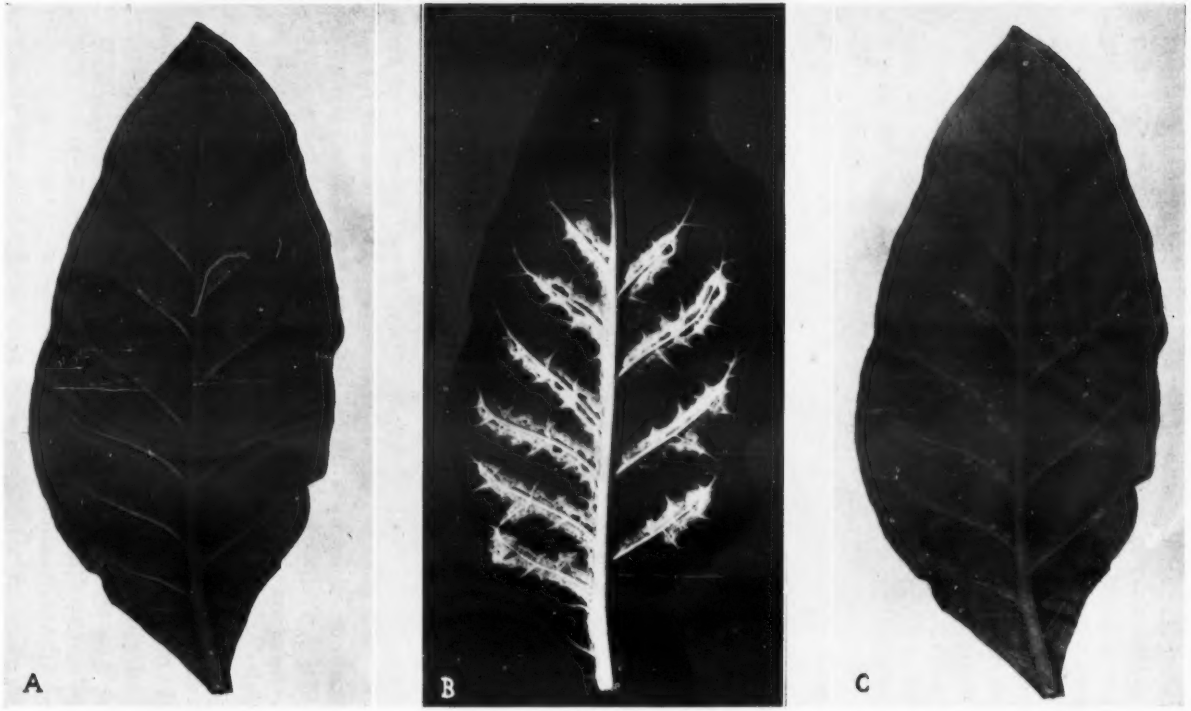

Fig. 3. Photographs of the under surface of a tobacco leaf showing systemic invasion by the virus of tomato spotted wilt.

A. Showing the necrosis on the under surface when photographed by daylight on a panchromatic plate. A Wrattan X2 filter was interposed between leaf and camera.

B. Showing fluorescence on the under surface when photographed under filtered ultra-violet light. An aesculine filter was placed between the leaf and the camera to absorb any reflected ultra-violet X rays.

C. As for A, except that a Wrattan A filter replaced the X2 filter.

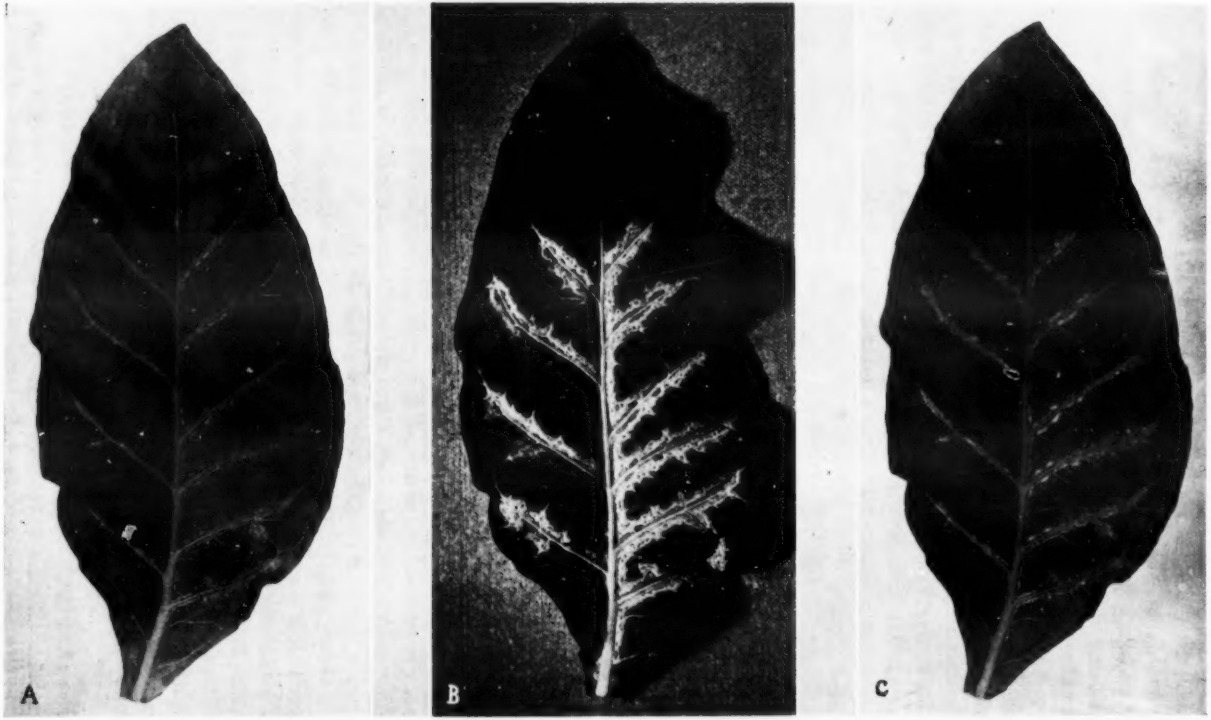

Fig. 4. Photographs of the upper surface of the same tobacco leaf as shown in figure 3.

A. Showing the necrosis on the upper surface when photographed by daylight on a panchromatic plate. A Wrattan X2 filter was interposed between leaf and camera.

B. Showing fluorescence on the upper surface when photographed under filtered ultra-violet light. An aesculine filter was placed between the leaf and the camera to absorb any reflected ultra-violet rays.

C. As for A, except that a Wrattan A filter replaced the X2 filter.

Later the fluorescent area extends outward, and is followed by necrosis until practically the whole leaf becomes necrotic. Pigmentation is not so pronounced as it is in the primary lesions, and shortly after death a large portion of the leaf (systemically infected) may still fluoresce, although not to the same degree as before. As was found in the case of primary lesions, the fluorescence is brighter on the under surface. In a number of places on 4B, and to a lesser degree on 3B, there is an alternation of dark and light lines. This same phenomenon is more clearly shown in the primary lesions produced by this virus in a native species of *Nicotiana*, where the alternations take the form of concentric circles. This phase will be dealt with more fully in a later communication in connection with a study of the ringspot character of primary lesions. Figures 3A and 4A represent the appearance of the necrosis as seen by the eye in daylight more nearly than Figs. 3C and 4C, but the latter are included to show the maximum contrast obtainable by using selective filters.

Application of the Effect to a Study of the Spread of a Virus in its Host.

The phenomenon may be applied to a study of the spread of a virus in the plant. The presence of active virus in leaves such as that shown in Figs. 3 and 4 is fairly evident from the effects produced, but has also been directly shown by using such leaves as a source of inoculum for infection of further plants. Although the effect is not confined to the one host or even to the one virus, its application for this purpose will be limited to those virus-plant combinations which produce the fluorescent substance in abundance. It may also conceivably happen that virus will enter a portion of a leaf without causing a fluorescence; and further, the fluorescent material may be transported in the vascular bundles without virus necessarily being present also. It has also this disadvantage in common with all other methods of estimating virus movement (and one which is often overlooked), namely, that it merely demonstrates the time of appearance of virus in certain parts of the plant. It does not necessarily follow that the part of the plant in which the virus is first present in demonstrable amount (except, of course, in the inoculated leaves) is the one to which it first moved. Different parts of the plant are more favourable for virus multiplication, and it is possible, and even probable, that virus entering two leaves simultaneously in equal amounts will find conditions more favourable for development in one than in the other, and may not, in fact, develop in the second sufficiently for its presence to be demonstrable (by any method) until much later.

However, the chief advantages of the new method are that the movement may be followed in the same plant over a long period without mutilating the plant in any way, and it is possible to trace details which cannot be determined by any other method at present in use. In the following discussion on the movement of the virus to and in the leaf shown in Fig. 3 the limitations discussed above should be kept in mind.

The leaf shown in Figs. 3 and 4 developed after the lower leaves had been inoculated. Following the usual procedure for quantitative work the plants were trimmed to two leaves per plant before inoculation. After inoculation, and while the primary

lesions were developing, some of the plants made strong secondary growth, and these were kept under observation for some time after the original inoculated leaves had died. At first the secondary leaves looked quite normal, but later a fluorescence (under ultra-violet light) was observed to extend some way along the midrib and later along the lateral veins at the proximal end, followed by a similar development along the other lateral veins in order, beginning at the proximal end and extending gradually to the distal end. The whole process covered a period of some days. The leaf shown in Fig. 3 had to be removed from the plant for photographing, and was therefore not followed further, but the general course of virus activity is as described in the previous section for the spread of the fluorescent areas.

It is fairly obvious that the virus has moved from the inoculated leaves to the new growth by way of the vascular bundle. Once in the leaf the movement appears to be along the midrib and out along the lateral veins. From the midrib and the lateral veins the virus apparently travels along the lesser veins, and also out into the mesophyll tissue (direct from the midrib, the lateral veins, and lesser veins), spreading slowly on both sides until eventually the whole of the area between adjacent lateral veins is invaded. In the leaf shown in Figs. 3 and 4 the fluorescence was first observed along the veins at the proximal end, and extended gradually to the distal end several days later. However, the fluorescence sometimes appears first along the veins at the distal end, followed later by its appearance along the veins nearer the base of the leaf. It is scarcely likely that the virus would enter a vein at the distal end before it did one near the base of the leaf, and it therefore follows that in this case the conditions were more favourable for virus multiplication at the distal end. It appears that the time taken for the virus to traverse the midrib is short in comparison with the time taken to spread into the mesophyll tissue, and that it is present in demonstrable quantity first in those parts of the leaf which are most favourable for its development or which are most susceptible to it. In which particular elements of the bundle the virus travels is more difficult to determine, but from our knowledge of the pH range over which this virus remains active, and from the pH value of the tissues, we may rule out the xylem.

It has been shown by Best and Samuel (1936) that T.S.W. virus is rapidly inactivated at a pH value of 5 and less. Our knowledge of the pH values prevailing in plant tissues rests on a very insecure basis, but it may be fairly concluded from Small's (1929) evidence that the pH value of the xylem in *N. tabacum* lies between 4 and 5, and that it is therefore very unlikely that the virus could exist there in an active state. This leaves the phloem as the most probable channel for the movement, although other elements are not excluded.

Examination of Other Hosts for T.S.W. Virus.

The account given in the preceding sections has dealt entirely with the interaction between T.S.W. virus and *N. tabacum*. In addition other hosts were examined to see whether the fluorescence phenomenon was a general one.

Lycopersicum esculentum.

In order to maintain a supply of T.S.W. virus, a fresh lot of *L. esculentum* plants (var. Dwarf Champion) are inoculated each week and kept in an isolated compartment of the glass-house. The symptom picture during the development of the disease in this plant has been fully described by Samuel, Bald, and Pittman (1930), and need not be repeated here.

Examination of the plants at various stages of the disease had failed to demonstrate the presence of the fluorescent material when plants were taken at random over a period of several months. Subsequently, however, on several occasions a few of the secondary necrotic lesions on the upper leaves did show fluorescent haloes surrounding a small proportion of the lesions. When, however, plants showing typical bronzed lesions on their upper leaves were decapitated, leaving only the middle and lower leaves, and these decapitated plants were left in the laboratory (in diffuse daylight) for several days, the middle leaves which had previously shown no symptoms of the disease developed large non-pigmented necrotic lesions. These lesions fluoresced brightly at their outer edges when viewed under ultra-violet light. It is worth noting that the leaves which develop the fluorescence are the more resistant ones from the point of view of secondary virus infection. The fluorescence has not been observed on the leaflets of healthy tomato plants, but blotting paper traces of pressed healthy leaves show very faint fluorescent lines along the mid-ribs of the leaflets. The amount is much smaller than that present in healthy tobacco plants.

Petunia hybrida.

Petunia leaves when artificially inoculated with T.S.W. virus develop primary lesions closely resembling those formed on tobacco leaves, except that there are usually only four or five lesions per half-leaf even when concentrated inocula are used. These primary lesions are surrounded by brightly fluorescent haloes when viewed under ultra-violet light, and what has been said of the primary lesions on tobacco leaves applies here also. The author has not observed a systemic infection of this plant, except possibly at the seedling stage.

Healthy petunia plants fluoresce brightly in various parts, from the seedling to the flowering stage, and in general much more brightly than healthy tobacco plants.

Solanum tuberosum.

Primary lesions of T.S.W. on potato leaves fluoresce at their outer edges. The fluorescence, which is of the same type as in tobacco leaves, appears, however, to be more easily destroyed by processes operating in the living leaf.

Tropaeolum majus.

Inoculated nasturtium leaves do not develop necrotic lesions but only diffuse yellow patches, and sometimes not even these are visible, yet the plant becomes systemically infected.

Examinations of healthy and diseased plants failed to demonstrate the presence of the fluorescent substance in any part of the plant.

Nicotiana glutinosa.

Primary lesions of T.S.W. on *N. glutinosa* are surrounded by a fluorescent halo of much the same character as those on tobacco, but the fluorescence is usually less intense and is spread over a wider area. The necrosed discs do not lose their fluorescence entirely, and when the leaf dies (on account of widespread necrosis) the whole leaf often shows a bright whitish fluorescence.

Examination of Plants Infected with Tobacco Mosaic Virus.

Leaves of *N. tabacum* systemically infected with ordinary mosaic virus show a faint blue fluorescence in the pale green areas of mottled leaves.

Primary lesions of tobacco mosaic virus on *N. glutinosa* leaves are surrounded by fluorescent haloes which are usually much less intense than, and never as extensive as, those described for T.S.W. on this plant. The relative intensity as compared with T.S.W. on tobacco can be judged from the photograph in Fig. 5, where A shows the appearance in daylight, and B under ultra-violet light. The exposure for B in Fig. 5 was 50 p.c. longer than for B in Fig. 1.

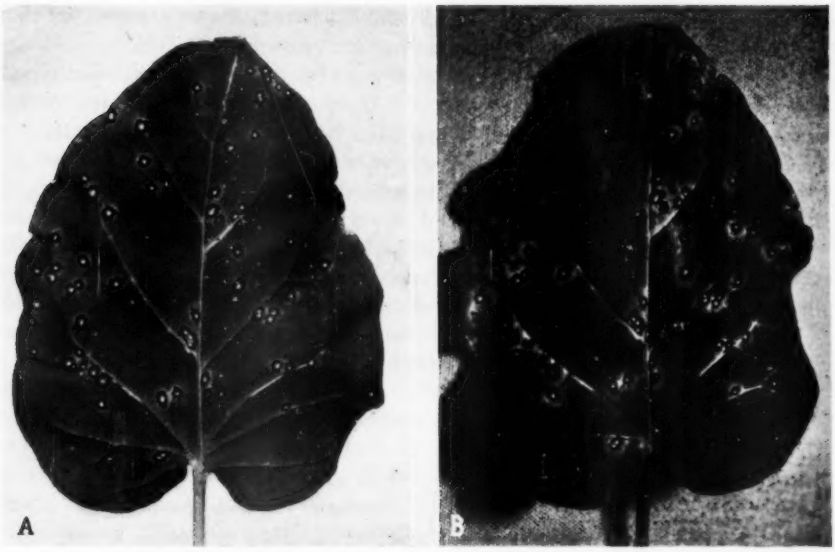

Fig. 5. Photographs of the upper side of a *Nicotiana glutinosa* leaf carrying primary lesions of tobacco mosaic virus.

A. Photographed in daylight on a panchromatic plate.

B. Showing the fluorescent haloes surrounding the lesions. Photographed under filtered ultra-violet light with a filter between leaf and camera to cut out the ultra-violet rays.

The fluorescence of the central spots is of a different character from that of the haloes.

No production of fluorescent substance has been observed in tomato plants systemically infected with this virus.

DISCUSSION.

Although more host plants will need to be examined before it is possible to generalize, the present data show a close parallelism between the amount of fluorescent substance normally present in the healthy plant and the type of infection under normal temperature conditions (local, local with severe systemic, mild local and medium severe systemic, or almost symptomless systemic). If we grade those

plants which have been examined, in accordance with the abundance of fluorescent substance in the healthy plant, we get the following series: petunia > tobacco > *N. glutinosa* > tomato > nasturtium. This is also the order of severity of the disease in these plants (considering a wholly local infection of petunias as being so severe that rapid destruction of the invaded tissues prevents the virus from spreading far).

There is also a connection of a different kind between the type of infection and the production of fluorescent material. In discussing this we need to take into account both viruses studied. Enhanced production of the fluorescent substance by T.S.W. is not confined to tobacco plants, but applies to other hosts also, and, moreover, the effect is not specific for T.S.W. virus. The substance accumulates as a result of injury, and the amount produced seems to be dependent on the type of injury and the amount of substance normally present in unaffected plants. A very severe injury results in less of the substance being produced than when the injury is only moderately severe. Mild injury causes least production of the substance. In the case of such plants as nasturtium, it cannot be decided without further evidence whether the non-appearance of the substance is due to the type of injury or to the apparent absence of the substance from healthy plants or to both.

In order to see whether the substance is produced as a result of mechanical injury and injury caused by other means, some experiments were carried out in which various parts of plants were injured in various ways. On account of the many complications associated with the estimation of the fluorescent substance in living plants by means of the brightness of the fluorescence, an account of these experiments will be held over until repeated, when better methods have been developed for estimating the substance. However, in the case of mechanical injury, it was quite clear that an enhanced fluorescence usually developed after some time at the site of the injury, provided that the injury was not too severe. Most significant of all is the fact that in decapitated healthy plants the substance accumulates in the stem and roots to an extent only surpassed by a similar accumulation in diseased plants. The occurrence of the substance in practically every part of healthy specimens of some species, even at the cotyledon stage, indicates that it is not formed only as a result of injury, but rather that more of it is present in the plant, at any one time, as a result of injury.

SUMMARY.

When a tobacco leaf with primary lesions of tomato spotted wilt (T.S.W.) virus is viewed under filtered ultra-violet light, the lesions are seen to be surrounded by brightly fluorescent haloes which extend into the apparently unaffected green mesophyll, and spread radially outward as the lesion develops.

The fluorescence is caused by a water-soluble, relatively stable organic substance which is present in small quantities as a normal constituent of healthy

tobacco plants, but is produced in much larger quantities as a result of virus activity.

Systemic invasion of uninoculated tobacco leaves by T.S.W. virus is shown by bright fluorescent areas, and the effect has been applied to trace the movement of the virus in the leaf. It is concluded that the virus travels to the leaf by way of the vascular bundles, into the midrib, along the lateral veins, and from these into the smaller veins, and from all these out into the mesophyll.

The fluorescent substance was also produced around primary lesions of tobacco mosaic and T.S.W. viruses on *N. glutinosa*, and around primary lesions of T.S.W. virus in petunia and potato leaves, rarely around secondary T.S.W. lesions on tomato leaves, and not at all in nasturtium leaves infected with T.S.W.

Healthy specimens of the plants mentioned above have also been examined for the fluorescent material, and a close parallelism appears to exist between the presence of this substance in unaffected plants and the type of infection resulting after inoculation with T.S.W.

Tobacco plants systemically infected with ordinary tobacco mosaic virus show only a faint fluorescence in the pale green areas of mottled leaves, and the leaves of tomato plants infected with this virus do not contain demonstrable amounts of the substance.

The substance is not developed as a result of virus infection of all host plants, and the reaction is not specific for T.S.W. virus, but appears to have a wider significance in connection with injury to the tissues.

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A BACTERIUM CAUSING "DISEASE" IN FORTIFIED WINES

by

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That wine, in common with many other organic solutions, may be the seat of microbiological activity, has been known since the time of Pasteur. Under certain conditions, the growth of micro-organisms and the end products of their metabolism may be such that the quality of a wine is seriously impaired, and the wine may become unfit for consumption: a wine so affected is said to be diseased.

Bacteria have been isolated from diseased wines and studied fairly intensively in Europe during the last half century. Bordas (1898) isolated and studied bacteria causing bitterness and sourness in wine. Laborde (1898 and 1904) studied the organisms isolated from wines showing different disease symptoms, and concluded that the nature of the disease, as indicated by the end products formed in wine, is governed by the nature of the wine, and by general conditions of environment, and not wholly by the species of the organism. This view has later been supported by Pacottet (1926) and by Ventre (1931). Müller-Thurgau and Osterwalder (1913) isolated and studied in considerable detail several species of bacteria producing lactic acid in wine.

Most of the literature on wine diseases published by the earlier European workers deals chiefly with diseases of unfortified wines, for wines of which the alcohol content had been raised to more than 17 p.c. by volume were seldom, if ever, attacked by micro-organisms. Recently, however, the occurrence of bacterial disease in wines of high alcoholic strength has been reported in different countries. D'Estivaux (1935) has recorded the finding of active bacteria in wine containing 18 p.c. by volume of alcohol, while Australian wine-makers have reported the occurrence of a bacterial disease in highly-fortified wines of which the alcohol content, in some cases, exceeds 20 p.c. by volume. It is generally believed by the wine-makers of this country that some wines are much more prone to this type of disease than are others of the same alcoholic strength. Evidence in support of this belief is found in the incidence of the disease, which cannot well be explained in terms of bacterial infection alone.

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[1] Research Officer of the Wine Overseas Marketing Board.

The object of this work, which was commenced early in 1935, was to isolate the causative organisms, to study their growth requirements, and especially to investigate the question of the susceptibility of fortified wines to disease. This report deals with the first section of the work, and comprises a description of an organism isolated from diseased wines, and its behaviour under cultural conditions.

MATERIALS AND METHODS.

Since the inception of this work, a number of samples of diseased wines and lees have been submitted for examination. The majority of these samples have been of the sweet white type, but several samples of sweet red wine and a few of fortified dry white wine showing bacterial growth, have also been received. The diseased wines examined are all characterized by a peculiar silky turbidity, an abnormally high volatile acidity, and a characteristic odour and taste which are more or less pronounced according to the severity of the disease. When examined microscopically, these wines are found to contain numerous micro-organisms of the rod type, usually joined in chains of various lengths.

Many of the samples received have proved to be unsuitable as sources from which to make isolations. This is probably due to the fact that in many cases a considerable period of time has elapsed since the disease first made its appearance. In some of these samples, the reduction of alcohol content by evaporation has been sufficient to allow a secondary infection by other organisms.

Of the several culture media used during the course of the work, the best results have been obtained with a medium which consists of:

Filtered autolysate of 110 gm. of pressed yeast diluted to 1 litre.	
Potassium di-hydrogen phosphate	1·0 gm.
Magnesium sulphate	0·2 gm.
Potassium hydrogen tartrate	3·0 gm.
Glucose	15·0 gm.
Sucrose	15·0 gm.

pH 4·5

Except where otherwise intimated, this medium has been used throughout the experiments described below. For plate cultures, the above-mentioned medium, solidified with agar or gelatine, has been used.

In order to ascertain the effects of different environmental factors on the growth of the organism, the increased titratable acidity of cultures, as compared with uninoculated controls, has been used to estimate growth quantitatively.

All determinations of hydrogen ion concentration of wines and of artificial media were made by means of the quinhydrone electrode.

EXPERIMENTAL.

Several organisms have been isolated from diseased wines, but so far only one of these has produced a vigorous growth when inoculated into a fortified wine. This organism has been isolated from wines of different types, and from different districts of South Australia.

Description of the Organism.

Morphology: rods ·4–·6 by 2–4 microns, occurring singly and in pairs, and often in long thread-like chains (long slender rods and long chains usually associated with old cultures in media of high alcohol content). Gram positive. Non-motile. Without endospores.

Gelatine stab: growth uniform from top to bottom, no liquefaction.

Gelatine colonies: subsurface, round creamy white.

Agar colonies: subsurface, creamy white to pale yellow, shape irregular, often angular and approaching stellate.

Agar slant: weak filmy surface growth under anaerobic conditions only.

Litmus milk: unchanged.

Potato slant: no growth.

Micro-aerophyllic.

Effect of the Bacteria on Wine.

From repeated failure to grow the bacterium in certain wines, it seems evident that only some wines are suitable for its growth. This is in accordance with the experience of South Australian wine-makers. The extent to which different wines are affected by inoculation with the bacterium is illustrated by the following experiment.

Three different wines of the same type (sweet white), and made in the same wine cellar, were inoculated with the organism, and after 10 weeks showed the following results:

Wine.	Result.
29	Unchanged, no bacterial growth.
40	Turbidity very marked. Titratable acidity increased by 3·2 gm. per litre, as tartaric acid.
42	Turbidity distinct but less marked. Titratable acidity increased by ·3 gm. per litre.

Some of the more important changes brought about by the bacterium in a sweet wine are shown in Table 1. A suitable sweet white wine, after passing through a Chamberland porcelain filter, was placed in sterilized 4 oz. bottles. Some of these were inoculated, and some were kept as controls. After remaining for 12 weeks at room temperature (20°–25° C.) the contents of these bottles were examined. A comparison of the analysis of the inoculated samples with that of the controls shows that the bacterium has caused a reduction in sugar and an increase in both fixed and volatile acidity. There is also an apparent reduction in alcohol content.

TABLE 1.

Effect of 12 Weeks' Growth in Sweet White Wine.

	Controls.	Inoculated.
Alcohol, per cent. by volume	19·0	16·8
pH	4·30	3·90
Titratable acidity		
Fixed acidity as gm./litre tartaric acid	3·80	4·85
Volatile acidity as gm./litre acetic acid	·36	2·15
Reducing sugar as gm./litre glucose	110·0	94·1
Condition	Bright	Very turbid
Microscopic examination	No bacteria	Bacteria very numerous

Behaviour of the Organism under Cultural Conditions.

The effects of some of the more important environmental factors on the growth of the organism have been investigated, and are shown in the following experiments.

Effect of Hydrogen Ion Concentration.

Quantitative determinations of the effect of pH on the growth of the organism have been made by inoculating artificial media after adjusting the pH with normal HCl and normal NaOH. All strains investigated show approximately the same optimum pH range, although the total range over which growth occurs appears to be slightly greater with some strains than others. Table 2 shows the effect of pH on the growth of the organism in an alcohol-free medium.

TABLE 2.

Four Days' Growth in Alcohol-Free Medium at 30° C.

pH	Increase in titratable acidity as g./l. tartaric acid.
3·0	—
3·4	—
3·8	1·27
4·2	2·25
4·6	3·60
5·0	3·60
6·5	1·00

The growth-retarding effect of pH values above or below the optimum range is more marked in media containing a high percentage of alcohol than in alcohol-free media. Table 3 shows the effect of pH on the growth of the organism in the standard medium, to which 10 per cent. by volume of alcohol had been added.

TABLE 3.

Growth in 10 p.c. Alcohol at 30° C.

	Increase in titratable acidity as g./l. tartaric acid.	
pH	21 days' growth.	42 days' growth.
3·0	—	—
3·4	—	—
3·8	·22	·50
4·2	1·98	4·00
4·6	3·36	5·50
5·0	·50	4·80
6·5	·22	1·20

The organism appears to have an optimum pH range of from about 4 to 5. The retarding effect of pH values above the optimum range is most obvious during the early stages of growth. As the medium used was less strongly buffered above pH 5·0 than below this value, the reduction of the pH of the medium caused by the activity of the organism gradually renders conditions more favourable for its development in these cultures.

Table 4 shows the influence of low pH values on the growth of the organism in a sweet white wine. Four-ounce bottles of wine were inoculated after adjusting the pH with citric acid and normal NaOH. After eleven weeks the increase in titratable acidity as compared with uninoculated controls was determined. It was found that under these conditions pH values below 3·8 seriously retarded the growth of the organism.

TABLE 4.

Effect of pH on Growth of Wine No. 40—11 Weeks at 23° C.

pH	Increase in titratable acidity as g./l. tartaric acid	Condition.
3·6	·10	Very slight turbidity
3·8	·70	Distinctly turbid
4·0	2·50	Very turbid
4·2	3·25	Very turbid and heavy deposit on bottom of bottle
4·4	3·70	

Effect of Temperature.

The effect of temperature on the growth of the organism was determined by incubating cultures in a multiple temperature incubator. The effect of different temperatures on growth in media of different alcohol contents is shown in Table 5. In an alcohol-free medium, some growth occurred at all temperatures between 14° C. and 36° C., after incubating for seven days. Under these conditions, the optimum temperature was in the vicinity of 32° C. In media of high alcohol content, growth was much slower at all temperatures. In media containing 15 per cent. by volume of alcohol, it was necessary to incubate cultures for 28 days in order to obtain a strong growth, while for the cultures in 20 p.c. alcohol the incubation period had to be extended to 56 days. Consequently, determinations of the increased titratable acidity of these cultures were made after a longer period of incubation than were those of the cultures in 10 per cent. alcohol and in alcohol-free media.

Source of Nitrogen.

The organism developed freely only in the presence of complex organic compounds. Autolysed yeast extract has proved the most satisfactory culture medium used in this laboratory. Some growth has also been obtained in haricot bouillon, malt extract, peptone medium, and marmite medium, but no growth occurred when simple salts such as nitrates or ammonium salts were the only form of nitrogen present. Analyses of a number of samples of the autolysed yeast extract medium used show that the total nitrogen content is approximately 0·2 p.c., of which approximately one-third is present as amino nitrogen, as determined by the method of Van Slyke.

TABLE 5.

Effect of Temperature on Growth in Different Concentrations of Alcohol.

Temp. °C.	Increase in titratable acidity as g./l. tartaric acid. Alcohol-free. 7 days' growth.	10 p.c. alcohol. 7 days' growth.	15 p.c. alcohol. 28 days' growth.	20 p.c. alcohol. 56 days' growth.
7	—	—	—	—
11	—	—	—	—
14	T	—	—	—
15·5	·43	T	—	—
18	1·07	·10	T	T
20	1·30	·23	·10	T
22	2·45	·38	1·00	·90
23·5	2·90	·53	1·60	1·55
25	3·21	1·15	·25	T
27	3·89	1·60	T	—
29	4·54	·99	—	—
32	5·01	T	—	—
36	4·35	—	—	—

— signifies no apparent growth.
T signifies slight turbidity produced but no measurable change in titratable acidity.

Fermentation Reactions.

For a study of fermentation reactions various sugars and organic acids were added to autolysed yeast extract. The fermentation reactions are summarized in Table 6. These reactions were the same for all strains investigated.

TABLE 6.

Fermentation Reactions.

Medium.	Reaction.
Yeast extract alone	No growth
do. + glucose	Acid and gas
do. + levulose	Acid and gas with production of mannite
do. + arabinose	No growth
do. + sucrose	Acid and gas
do. + lactose	No growth
do. + raffinose	Acid and gas: very slow growth
do. + mannite	No growth
do. + malic acid	No growth
do. + potassium hydrogen tartrate	No growth

DISCUSSION.

Systematic Relationships.

The morphological and cultural characters of the organism as described above place it in the genus *Lactobacillus*, as described by Bergey (1934).

Certain cultural characters, however, distinguish the local organism from the species described by Bergey as occurring in wine. Its failure to ferment arabinose

and lactose distinguishes it from *Lactobacillus mannitopœus*, *L. intermedius* and *L. gayonii*, while its failure to ferment malic acid and its high alcohol tolerance distinguish it from *L. gracilis* (*Bacterium gracile*) as described by Müller-Thurgau and Osterwalder (1913). The local organism resembles that described by D'Estivaux (1935) in its high alcohol tolerance, but differs from it in the production of mannite from levulose, and also in its temperature relationship.

Cultures of the local organism have been grown in artificial media and in wine containing 20 p.c. by volume of alcohol, but it is not supposed that this figure represents the extreme limit of the organism's tolerance to alcohol. Though some of the strains under observation appear to have a higher alcohol tolerance than do others, sufficient data on this point have not yet been accumulated to warrant the assumption that strains differ significantly in this respect.

It has been shown that wines vary greatly as regards their susceptibility to disease caused by bacteria such as that described above. This important question of susceptibility is at present not fully understood, and is the subject of further investigation.

SUMMARY.

A bacterial disease occurring in fortified wines has been studied. An organism has been isolated from diseased wines of different types made in different districts of South Australia, and some of the effects produced by this organism when grown in wine have been determined.

The effects of certain environmental factors on the growth of the organism have been investigated with a view to ascertaining optimum and limiting conditions with respect to each factor. The optimum pH range lies between 4 and 5, while a hydrogen ion concentration greater than that represented by pH 3·8 seriously retards the growth of the organism in wine or in artificial media. The organism has a high alcohol tolerance, and the effect of incubation temperature varies with the alcohol content of the medium. The chief morphological and cultural characters of the organism are described, and its systematic relationship to organisms described by other workers is discussed.

ACKNOWLEDGMENTS.

The author wishes to acknowledge his indebtedness to Professor J. A. Prescott, of the Waite Agricultural Research Institute, under whose direction the work was carried out; to the Australian Wine Overseas Marketing Board, on behalf of whom the problem is being investigated, for permission to publish this report; and to those wine-makers of South Australia who have supplied information and samples of wine and other materials.

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THE EFFECT OF LIGHT AND TEMPERATURE ON THE DEVELOPMENT OF PRIMARY LESIONS OF THE VIRUSES OF TOMATO SPOTTED WILT AND TOBACCO MOSAIC[1]

by

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INTRODUCTION.

The effect of differences in environmental conditions on the symptom picture of various plant virus diseases has been described by a number of workers, and a good summary is to be found in Kenneth Smith's monograph (1933). Such studies have included the effect of conditions prevailing during the developmental period, on the nature of primary lesions, and Shapovalov and co-workers (1930, 1931) have found that shading reduced the percentage of tomato plants which became systematically infected with tomato yellows. Except for a note by the author (1935), there appears to be no record of the effect of differences of environmental factors, subsequent to inoculation, on the actual number of primary lesions produced. A knowledge of such effects is essential to a proper interpretation of infectivity data obtained by the primary lesion method, particularly when the change of virus activity of a given suspension over time periods extending beyond a few hours is being investigated. Such knowledge may also be expected to add to our understanding of the interactions between plant and virus and of the virus itself.

Normally, inoculated plants are left in the glasshouse until the lesions develop to a number and size suitable for accurate counting. Only a small measure of control over temperature, light intensity and amount, and humidity is thus possible. In order to form some idea of the effect of variations in these factors under normal working conditions, the rate of appearance of lesions and the total number resulting from the same inoculum were determined when comparable lots of inoculated plants were exposed to the conditions prevailing in the laboratory on the one hand and in the glasshouse on the other. These experiments, besides furnishing the information required, indicated the importance of temperature and light.

[1] Glasshouse facilities and part of the cost of these investigations are being provided by the Council for Scientific and Industrial Research (Australia).

Further experiments were therefore carried out in cabinets under controlled conditions of light and temperature. It was not found possible to control the humidity effectively. The viruses studied were ordinary tobacco mosaic virus, inoculated on leaves of *Nicotiana glutinosa*, and tomato spotted wilt virus, inoculated on leaves of *Nicotiana tabacum* (var. Blue Pryor).

EXPERIMENTAL METHODS.

The choice, arrangement, and handling of test plants were as previously described (Samuel, Best, and Bald, 1935; Best and Samuel, 1936). The same applies to the preparation of inocula except that the suspensions were much more dilute with respect to infective juice, and in the chief experiments with tobacco mosaic a purified preparation obtained by precipitating the virus complex at its iso-electric point (Best, 1935; 1936a), and resuspending it in buffer solution, was used. All inocula were buffered at pH 7, and those of spotted wilt virus were protected by the presence of sulphite.

Test plants were removed to the laboratory twenty-four hours before inoculation. For any one experiment all plants were drawn from the same batch; they were inoculated under the same conditions at the same time with the same inocula.

All experiments carried out at constant temperature were performed in cabinets measuring $39 \times 36 \times 42$ inches. Artificial light was supplied by four 500 watt Mazda lamps per cabinet. The lamps were placed outside the cabinet at a distance of 3 ft. above the level of the leaves. The light intensity was found by means of a photometer to be 800 foot candles at the level of the middle leaves for *N. glutinosa*, and of both leaves for *N. tabacum*.

After inoculation the leaves were inspected at frequent intervals, and unless otherwise stated the lesions were counted when the first clearly visible ones appeared, and thereafter at suitable intervals.

EXPERIMENTAL RESULTS.

Glasshouse versus Laboratory Conditions.

During hot weather the temperatures in the glasshouse are frequently too high for lesion development, whereas lesions develop very well in the laboratory. It was therefore of some importance to obtain a comparison of the number of lesions developed and their rate of development under the two sets of conditions when both were favourable for lesion production. Table 1A shows the number of tobacco mosaic virus lesions which developed on *N. glutinosa* plants kept wholly in the laboratory or wholly in the glasshouse or partly in each. After the first appearance of lesions, pilot counts were made from time to time, and the counts recorded in Table 1A were made when it was evident that lesion appearance had practically reached an end point.

It will be seen that many more lesions developed on plants exposed to glasshouse conditions for the whole of the post-inoculation period than on plants exposed wholly to the conditions prevailing in the laboratory, and that an intermediate number developed on those plants which were kept in the laboratory for a short period followed by a longer period in the glasshouse. In addition to significant differences in the total number of lesions between glasshouse and laboratory

plants, there were differences in the ratio of the number of lesions for the two concentrations. In no case was this ratio equal to the ratio of the concentrations of virus, but this latter was approached more nearly under laboratory conditions in this experiment.

TABLE 1.

Showing the Effect of Conditions to which the Plants were Exposed after Inoculation, on the Number of Primary Lesions Produced.

A. *Tobacco mosaic virus on N. glutinosa.*

Time left after inoculation		No. of lesions: (a) on 16 leaves. (b) on 32 leaves.		
In laboratory.	In glasshouse.	1/200*	1/1000*	Ratio.
(a) 5 days	—	391	113	0·29
1½ days	3½ days	375	123	0·33
—	5 days	565	304	0·54
		1/1000*	1/5000*	
(b) 4 days	1 day	1,502	498	0·33
¾ day	4¼ days	1,937	577	0·33
—	5 days	2,381	895	0·38

* Concentration of inocula with respect to infective juice.

B. *Tomato spotted wilt virus on N. tabacum.*

Time left after inoculation		No. of lesions on 32 half-leaves* days after inoculation.		
In laboratory.	In glasshouse.	3	4	5
—	Entirely	639	797	831
Entirely (3 days)	—	805	—	—
3 days	1 day	—	1,285	—
3 days	2 days	—	—	1,346

* Concentration of juice in inoculum = 1/50.

Temperatures were much more uniform in the laboratory than in the glasshouse, but the mean temperature over the whole of the period from inoculation to final count was higher in the glasshouse.

In other experiments progressive counts, performed each morning and evening over a period of some days, of the lesions appearing under glasshouse conditions revealed that the rate of appearance was greater during the day than during the night, but the differences were not great, and therefore appeared to be due to the lower temperature prevailing at night.

In Table 1B are recorded the results of an experiment with tomato spotted wilt (T.S.W.) virus on *N. tabacum* carried out on lines similar to those described above for tobacco mosaic virus, except that only two sets of plants (trimmed to two leaves per plant) were concerned, and these were arranged at random into two 4 x 4 Latin squares. Actually the experiment was arranged to give two dilution

curves, the details of which will be reported later, but for the present purpose we shall consider only the total number of lesions developed on the 32 half-leaves of each set, which were inoculated with the common control suspension. One set was transferred to the glasshouse within fifteen minutes of completing the inoculations, and the other set was allowed to remain in the laboratory for three days, and was then transferred to the glasshouse[2]. At the first count (three days after inoculation) the laboratory plants had developed a higher number of lesions than the glasshouse plants. The difference became even greater by the fourth and fifth days, notwithstanding the transference of the laboratory plants to the glasshouse at the time of the first count. This experiment was performed in May, 1934, when the mean temperature in the laboratory was about 5° C. lower than that in the glasshouse.

Subsequently, in a number of experiments, lesions developing on inoculated plants in the glasshouse were counted twice daily (morning and evening) over a period of several days. The rate of appearance of lesions is shown for a typical experiment in Fig. 1. This experiment was performed when daylight periods were short and light intensity was relatively low, but even under these conditions it is evident that the rate of appearance of lesions was greater during the period of darkness, and that no new lesions at all appeared during the daylight period between the second and third counts. The first count (179 lesions) was made in the afternoon of the third day after inoculation. Actually most of these lesions were visible in the morning of the same day, but an attempt to count them accurately was abandoned on account of their smallness and indefiniteness.

It is appropriate at this stage to note the *character* of the lesions. Lesions which make their appearance overnight are less heavily pigmented than those which appear during the day. The pigmentation of lesions which develop in the subdued light of the laboratory was only just perceptible, and the lesions were larger and tended to spread more rapidly. These remarks apply to both virus-host combinations studied, but more so to T.S.W. virus on *N. tabacum*. A similar difference in the character of lesions developed in the glasshouse on covered as against uncovered sides of the same leaves, has been recorded by Samuel and Bald (1933), but no difference in the number of lesions was observed by them under their conditions. The extent of the pigmentation of the lesions which make their appearance during the night under glasshouse conditions appears to be determined by the light intensity of the preceding days, being faint if these have been dull, and more pronounced if these have been sunny. Further, although the appearance of new T.S.W. lesions is sometimes totally inhibited by sunlight, established lesions do spread during daylight, but to a less extent than during the night. This, coupled with differential pigmentation, often gives the lesions the

[2] Transference to the glasshouse at this stage was necessary because at such times of the year when temperatures are not too high in the glasshouse for good lesion development, the light intensity in the laboratory is such that lesions developed there spread so rapidly that later counts are difficult, and often of doubtful accuracy.

appearance of concentric bands in which narrow dark bands alternate with wider and more lightly-coloured ones. Apparently pigmentation is dependent on the presence of a product of photosynthesis which is used up in the dark, and the natural assumption to make is that this product is starch, or some derivative or breakdown product of it.

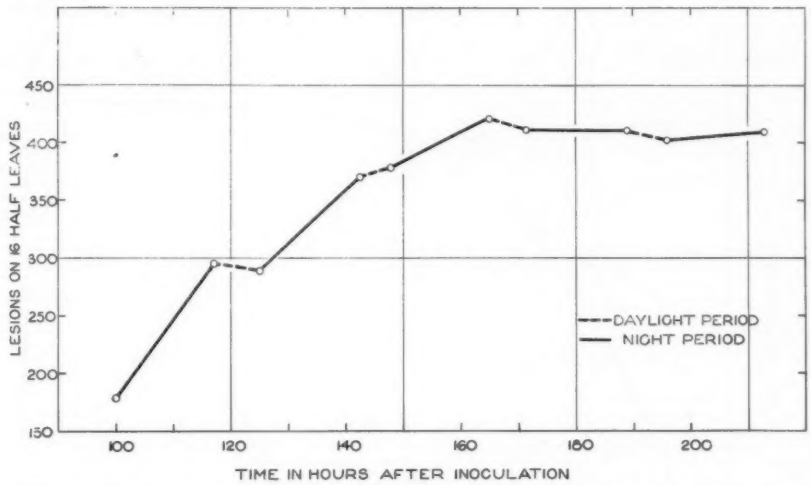

Fig. 1. Showing the rate of appearance of primary lesions of tomato spotted wilt virus on leaves of *Nicotiana tabacum* during daylight and night periods in the glasshouse.

The Effect of Light on the Rate of Appearance of Primary Lesions and on the Final Number Developed when the Temperature was Constant.

(a) *Tobacco Mosaic Lesions on N. glutinosa.*

A batch of six *N. glutinosa* plants trimmed to four leaves per plant was placed immediately after inoculation into a cabinet maintained at 20° C. The plants were exposed to alternate 12-hour periods of artificial light and darkness. The first lesion count was made during a "light" period $51\frac{3}{4}$ hours after inoculation, and thereafter at the times of transition from light to darkness and *vice versa*. The results are plotted in Fig. 2. From the smoothness of the curve we conclude that light of the quality and intensity used had no observable effect on the rate of appearance of tobacco mosaic virus lesions on *N. glutinosa*.

(b) *Tomato Spotted Wilt Lesions on N. tabacum.*

Experiments similar to those illustrated in Fig. 1 have been carried out on a number of occasions with the same results, and it may be concluded from these that sunlight exerts an inhibiting effect. This conclusion is further supported by the

fact that whenever a reasonable number of lesions appeared during a daylight period, this was either associated with a very dull day (a heavy dust-storm lasting all day on one occasion) or occurred during the early stages of lesion development, when the final count was very high. Even in such cases the rate was lower during the day than during the succeeding night. The temperatures were actually more favourable for lesion development during the daytime.

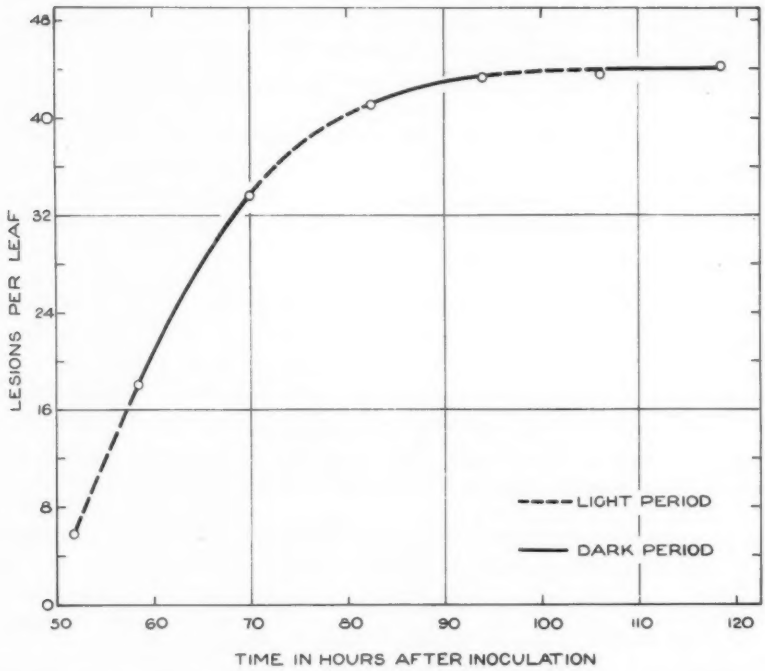

Fig. 2. Illustrating the rate of appearance of primary lesions of tobacco mosaic virus on leaves of *Nicotiana glutinosa* during alternating periods of darkness and artificial light in a cabinet at 20°C.

Attempts were made to reproduce these effects with artificial light in cabinets maintained at 20° C. An experiment in which a batch of plants was exposed to continuous illumination and another to alternate periods of light and darkness gave inconclusive results. The continuously illuminated batch developed only 65 lesions, as against 114 by the other. On account of the large variation in lesion numbers from leaf to leaf, this difference was not significant when tested by the "t" test. There were also no significant differences between light and darkness periods. A further experiment was performed in which 32 tobacco plants were

inoculated with the same suspensions of T.S.W. virus. They were divided into two sets of 16 plants each, and were transferred immediately after inoculation to two cabinets maintained at 20° C. One set of plants was exposed to continuous illumination. The other set was allowed to remain in complete darkness for 26 hours after inoculation; it was illuminated for two hours on the second day, and thereafter for 6 hours per day until the end of the experiment. The results of this experiment are summarized in Table 2 and Fig. 3.

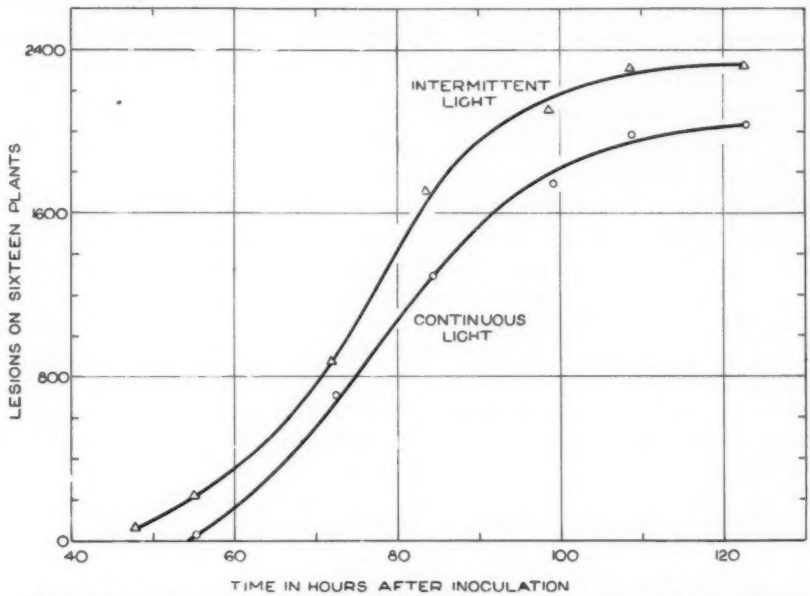

Fig. 3. Comparison of continuous and intermittent artificial lighting on the production of primary lesions of tomato spotted wilt virus on leaves of *Nicotiana tabacum* in cabinets maintained at 20°C.

TABLE 2.

Showing the Effect of Artificial Light on the Production of Primary Lesions of Tomato Spotted Wilt Virus on N. tabacum at 20° C.

Continuous light (800 foot candles).		Intermittent lighting.	
Hours after inoculation.	Lesions on 32 leaves.	Hours after inoculation.	Lesions on 32 leaves.
32	0	32	0
48	0	48	63
55·3	30	54·9	213
72·6	707	71·9	877
84·4	1,291	83·5	1,707
99·2	1,738	98·7	2,104
108·1	1,985	108·7	2,313
122·1	2,036	122·7	2,328

The effect of the extra light received by the one set was threefold. It caused a delay in the appearance of the first lesions and of the maximum in the rate curve, and further, it lowered the total number of lesions finally developed. Although new lesions were appearing up to the time of the last count, the limits to which the two summation curves are approaching are different, and are estimated approximately at 2,400 lesions for the intermittently illuminated set, and at 2,100 for the set under continuous illumination. Actually, the difference between the two means at the final count was found to be not significant, but the fact that in both experiments carried out with artificial light the effect was in the same direction, leads one to the conclusion that the effect is real but very much smaller than was found for daylight.

The Effect of Temperature on the Rate of Appearance and the Final Number of Lesions when the Light Factor was Constant.

(a) *Tobacco Mosaic Lesions on N. glutinosa.*

Thirty-two *N. glutinosa* plants trimmed to four leaves per plant were divided into two sets of 16 plants at random. One half of each leaf was inoculated with a suspension of purified tobacco mosaic virus equivalent to a concentration of one volume of infective juice in 500 volumes of inoculum, and the opposite half was inoculated with a similar suspension diluted to half this concentration. The concentrations of the inocula with respect to virus precipitate were approximately 6 and 3 parts respectively by weight in 10^6 parts of suspension medium. Plants were taken for inoculation alternately from each batch, and after allowing two hours for the leaves to dry (to avoid contamination during transfer) they were removed to two cabinets, one of which was maintained at 15° C. and the other at 20° C. Both sets of plants were exposed to continuous illumination. The results of the lesion counts are summarized in Table 3 and Fig. 4.

TABLE 3.

Showing the Effect of Temperature on the Rate of Appearances and the Final Number of Primary Lesions of Tobacco Mosaic Virus on N. glutinosa when the Light Factor was Constant.

15°C.		20°C.	
Hours after inoculation.	Lesions on 64 leaves.	Hours after inoculation.	Lesions on 64 leaves.
—	—	46·0	0
50·75	0	50·75	1,096
61·05	58	60·5	1,946
71·8	422	72·4	2,336
81·4	968	82·2	2,649
93·8	1,441	95·0	2,771
104·4	1,744	103·8	2,82
118·7	1,928	117·9	2,850
129·3	2,052	—	—
141·5	2,142	142·8	2,886
153·5	2,211	—	—
172·5	2,253	173·1	2,932

Lesions appeared earlier at the higher temperature, and the rate of appearance was greater at 20° than at 15° C. The time of appearance of the first lesions could be determined only to within a few hours. At an inspection 46 hours after inoculation, no lesions were visible on any plant, but 49 hours after inoculation a large

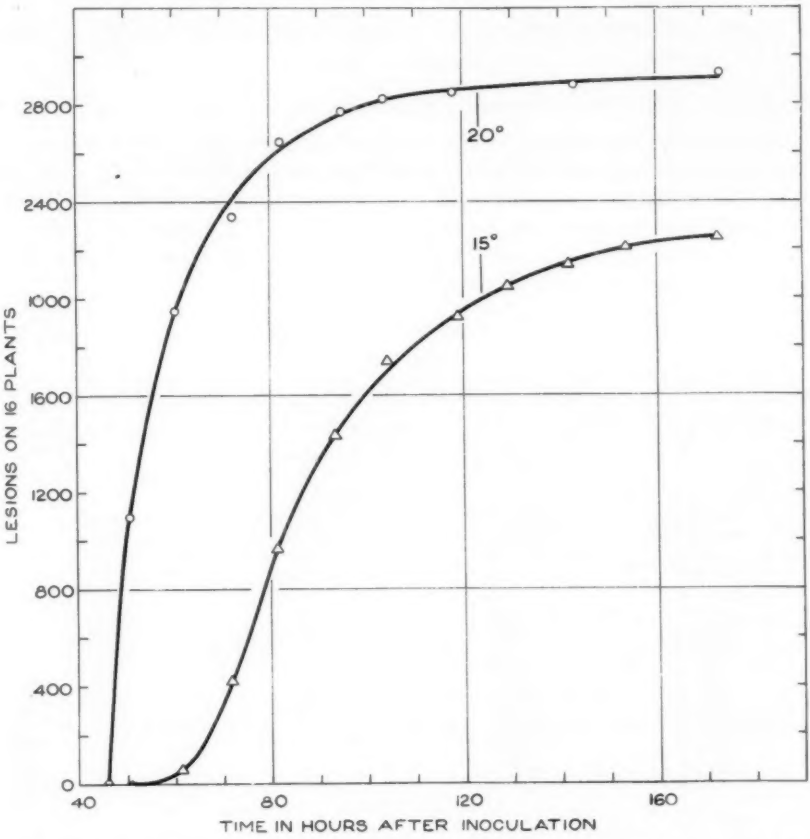

Fig. 4. The production of primary lesions of tobacco mosaic virus on leaves of *Nicotiana glutinosa* at 15°C. and 20°C. when the light factor was constant.

number of lesions was visible on the batch at 20°, and counting was begun about one-and-a-half hours later. The first count, which was half completed $50\frac{3}{4}$ hours after inoculation, revealed 1,096 lesions (17 per leaf). No lesions were then apparent on the 15° batch, but 10 hours later 58 lesions (0·9 per leaf) had appeared.

Taking into account the difference in the rate of lesion appearance, we may place the time of first appearance of lesions roughly at 47 and 56 hours after inoculation for the 20° and 15° batches respectively.

The most important difference, however, is that the total number of lesions developed is significantly higher at 20° than at 15°. At the final count, 30 p.c. more lesions had developed at 20° than at 15°, an increase of 6 p.c. per 1° C. The 15° curve approaches a limiting value of approximately 2,300, and the 20° curve a limit of approximately 2,950, an increase of 28 p.c.

(b) *Tomato Spotted Wilt Lesions on N. tabacum.*

Thirty-two tobacco plants trimmed to two leaves per plant were inoculated by the half-leaf method with two suspensions of unpurified T.S.W. virus. The concentrations of the inocula with respect to infective juice were 1 in 75 and 1 in 150. The handling of the plants was in every way similar to that described in the previous section for *N. glutinosa* plants inoculated with mosaic virus. The results of progressive counts are summarized in Table 4 and Fig 5.

TABLE 4.

Showing the Effect of Temperature on the Rate of Appearances and the Final Number of Primary Lesions of Tomato Spotted Wilt Virus on N. tabacum when the Light Factor was Constant.

15°C.		20°C.	
Hours after inoculation.	Lesions on 32 leaves.	Hours after inoculation.	Lesions on 32 leaves.
53·4	0	53·4	0
65·0	3	65·0	369
80·2	21	71·2	597
90·3	88	79·6	1,624
102·1	365	89·6	2,113
116·75	861	101·3	2,798
125·45	1,165	117·25	3,188
138·45	1,508	137·75	3,370
149·65	1,865	162·75	3,442
162·15	2,154	—	—
185·75	2,444	—	—
209·7	2,606	—	—
235·0	2,659	—	—

The first lesion count was made 65 hours after inoculation. This may be taken as the time of appearance of the first lesions for the 15° batch. Extrapolation to zero lesions may be made with fair accuracy for the 20° curve, and falls at about 54 hours. However, since no lesions were visible at an inspection made at this time, the lesions must have become visible very shortly after, and we may take 10 hours as the difference between the times of first appearance of lesions at the two temperatures.

Both curves approach a limiting value which is very different in the two cases, being approximately 2,700 for 15° and 3,500 for 20°, a difference of 29·6 p.c. At the final count, the total number of lesions which had appeared was 2,659 at 15° and 3,442 at 20°, an increase of 29·4 p.c., or 6 p.c. per 1° temperature difference. The difference was again significant.

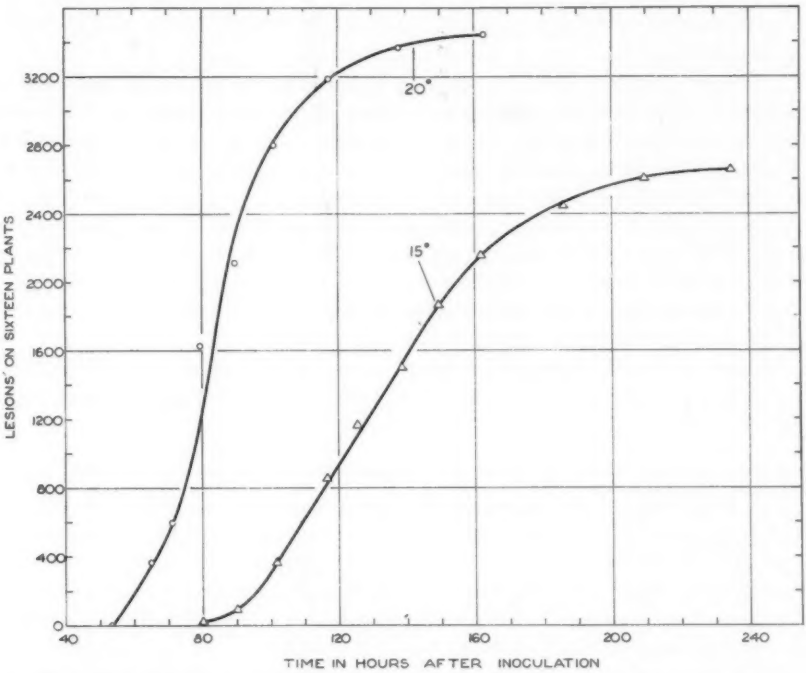

Fig. 5. The production of primary lesions of tomato spotted wilt virus on leaves of *Nicotiana tabacum* at 15°C. and 20°C. when the light factor was constant.

DISCUSSION.

The Sigmoid Form of the Summation Curves.

All the summation curves are sigmoid in form except that for mosaic at 20° C., and in this case lesion development was so rapid that a sigmoid portion would easily be missed. Each point on any curve represents the total number of lesions at any one time on a number of leaves on a number of plants, and if the rate of appearance of lesions varied considerably from plant to plant or leaf to leaf, and if the time of maximum development were distributed about a mean or modal

value, we would expect to get a sigmoid curve. Although neither every leaf nor every plant goes through its maximum at the same time, the variation was surprisingly small. Furthermore, when the progressive counts were plotted separately for each leaf, the sigmoid form was found to be a characteristic of the single leaf. This form for the single leaf would be produced if either or both of the following held: (1) If the degree of susceptibility of susceptible areas on the leaf surface is distributed about a modal value, and (2) If there exists in the inocula a size distribution of virus aggregates about a modal value, as is the case for hydrophylic sols in general.

This latter presupposes that the bigger the infective unit (with respect to the number of virus particles), the sooner (other things being equal) will the virus multiply to a stage where visible necrosis occurs. That all parts of a leaf are not equally susceptible is shown by the fact that with reasonably dilute inocula the majority of the lesions which develop are situated close to lateral veins, and the greater number of those which are not are formed near to or at the junction of the larger of the lesser veins, whereas with more concentrated inocula lesions appear more or less uniformly over the leaf surface. In the former case lesions appear equally on both sides of the lateral veins, so that a purely mechanical explanation for the distribution is very unlikely.

Since hydrosols in general are rarely monodisperse unless specially prepared or fractionated, the existence of a size distribution of virus aggregates is highly probable.

The Rate of Appearance of Primary Lesions as Affected by Temperature.

In Fig. 6 the rate of appearance of lesions is plotted against the time. It is clear from these that both the rate and time of appearance of the first lesions are dependent on temperature. Comparisons may best be made by considering the times at which the rate of appearance of lesions is a maximum. This time lies close to, but does not correspond exactly with, the time at which half the limiting number of lesions have appeared.

The times of maximum rate of appearance of lesions, together with the approximate times of their first appearance, are tabulated below.

	Tobacco mosaic virus.		Tomato spotted wilt virus.	
	20°C.	15°C.	20°C.	15°C.
Maximum rate of lesion appearance	51*	81	80	120
First appearance of visible lesions	47	56	55	65
Difference	4	25	25	55

* The numbers represent the time in hours after inoculation.

A drop of 5° C. caused a 60 p.c. increase in the time of the maximum rate and a difference of about 9 hours in the time of appearance of the first tobacco mosaic lesions.

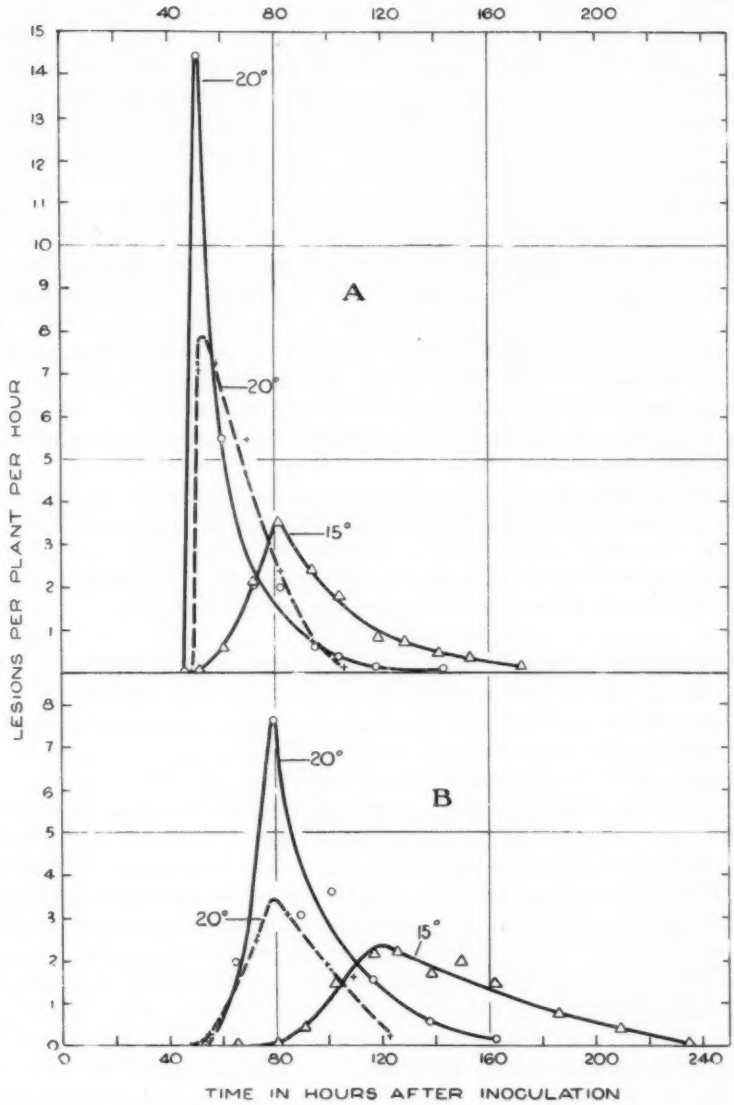

Fig. 6. Showing the rate of appearance of primary lesions at 15°C. and 20°C. when the light factor was constant. A. Tobacco mosaic virus on *N. glutinosa*. B. Tomato spotted wilt virus on *N. tabacum*.

For T.S.W. virus a difference of 5° C. caused a difference of about 10 hours in the time of appearance of the first lesions and a 50 p.c. increase in the time of maximum rate of appearance. Compared with tobacco mosaic, a much longer time elapsed between the first appearance of lesions and the attainment of the maximum rate. The time of maximum rate (from inoculation) is 60 p.c. longer for T.S.W. than for tobacco mosaic at 20° C., and 50 p.c. longer at 15°, a difference of 39 hours at the latter temperature.

The dotted curve of Fig. 6A was drawn from the data of Fig. 2, representing an experiment also carried out at 20° C. with tobacco mosaic virus on *N. glutinosa*, but performed fourteen months before the experiment represented by the 20° C. continuous curve. The dotted curve of Fig. 6B represents an experiment with T.S.W. virus on *N. tabacum* carried out at 20° C. a fortnight before that represented by the continuous 20° C. curve. It appears that for each virus-plant combination, the time interval between inoculation and the time of maximum rate of appearance of lesions is constant, and is little influenced by the condition of the plants. The time of first appearance of lesions does vary somewhat, depending on the final number developed.

Since the time of maximum rate at any one temperature is so different for the two virus-host combinations, this relationship may be useful in characterizing a system. Whether it is essentially a property of the virus or of the combination is not known, but T.S.W. lesions take much longer than mosaic lesions to appear on *N. glutinosa* leaves inoculated with a mixture of the two viruses.

The Effect of Light and Temperature on the Total Number of Lesions Developed.

In the case of T.S.W. virus on *N. tabacum*, the inhibiting effect of daylight on lesion appearance, and the lower totals obtained under continuous artificial illumination as against intermittent illumination, may be due to either or both of the following causes. Firstly, it is probable that a photochemical action results in some virus "colonies" being inactivated before the virus in them has developed to a sufficient concentration to produce visible lesions. The author (1935, 1936b) has recorded the presence in the tissues surrounding the lesions of a substance which fluoresces in ultra-violet light. Initially colourless solutions of the fluorescent substance develop a yellow colour on exposure to daylight, and exposure to the near ultra-violet rays of filter paper impregnated with the substance caused a marked reduction in the intensity of the fluorescence. It is likely that the difference in energy between the absorbed and emitted light is utilized *in vivo* in the production of intermediate products which take part in a reaction or series of reactions leading either directly or indirectly to the inactivation of the virus. This would account for the action of sunlight in retarding or inhibiting the appearance of lesions, and also for the difference in efficiency in this respect between sunlight and artificial light. Secondly, it has been shown (Best and Samuel, 1936b) that the virus of T.S.W. is inactivated *in vitro* by oxidizing systems which induce in the suspensions a redox potential above a certain limiting value (roughly an Eh

value of + 0·2 volt at pH 7), whereas oxidizing systems which induce a potential of + 0·1 or less at pH 7 do not in general inactivate the virus. Under the same conditions, reducing systems which induce a redox potential of + 0·1 volt or less protect suspensions, exposed to the air, against inactivation. More recently the author has been able to maintain suspensions of T.S.W. virus in an active state for 35 days at 0·5° C. by addition of cystein and exclusion of oxygen. It is generally accepted that when respiration exceeds photosynthesis, as in subdued light or darkness, the oxygen tension in plant cells is much lower than it is when photosynthesis exceeds respiration. To what extent, and how these factors will affect the virus of T.S.W. is not known, but it is very likely that the relatively more intense reducing conditions prevailing in the cells at low light intensities or in darkness will be more favourable for virus multiplication *in vivo* than at higher light intensities.

The difference between the total number of lesions developed at 15° C. and at 20° C. for both virus-host combinations is another matter. From the nature of the curves, the effect seems to be of a similar nature in both cases. On account of the arrangement of the experiments and the technique employed, the number of infective units which entered each batch as a whole, and therefore the number of potential lesions, must have been the same. It is clear that a greater proportion of the infective units entering the leaves have failed to develop into lesions at 15° C. than at 20° C. This difference may be connected with differences in the rate of protoplasmic streaming with temperature. Virus particles would thus be transported more rapidly at the higher temperature to localities favourable for multiplication. However, this alone would not explain the difference in the final number of lesions developed unless processes leading to inactivation or immobilization of the virus were also operating. The chances of any particular particle or aggregate reaching a locality suitable for rapid multiplication before inactivation occurred would then be greater at 20° C. than at 15° C. So little is known about the processes which operate even in a normal healthy leaf that a solution of the problem will have to await further advances in plant biochemistry. But whatever the mechanism may be, we may conclude that a minimum or critical number of virus particles is necessary for the production of a lesion, and that the value of this critical unit varies from plant to plant, from leaf to leaf, and from point to point of the same leaf.

The evaluation of even the relative amount of virus which enters at any one spot is not at present possible, and in consequence the concept of an average infective unit will be more useful in practice. Ideally, the average infective unit may be defined as either the weight or number of virus particles which enters or is adsorbed to a leaf per lesion produced. Even where the actual amount of virus concerned is not known, such a unit may be expressed in relative terms, as for example, when the same amounts of inoculum have been applied to two uniform batches of plants. However, in view of the difficulty of determining the actual quantity, it will be necessary to take as a working unit the amount of virus applied per leaf or half-leaf per lesion produced. The ideal value would be smaller than this, but proportional to it.

In the experiment reported in Table 3, the control inoculum contained approximately 3×10^{-6} gm. of tobacco mosaic virus complex per millilitre, and the amount of inoculum remaining on a half-leaf before washing off the excess is 0·1 ml. on an average. The weight of virus applied per half-leaf was therefore 3×10^{-7} gm., and this produced an average of 20·9 lesions at 20° C., and an average of 15 lesions at 15° C., so that the average infective unit was $1{\cdot}43 \times 10^{-8}$ gm. at 20° C., and $2{\cdot}0 \times 10^{-8}$ gm. at 15° C., a difference of 40 p.c.

In the case of T.S.W. inocula, we are not able to estimate the average weight of virus per lesion, but a relative value may be calculated as indicated above. It is found that for T.S.W. virus on *N. tabacum* the average infective unit is approximately 30 p.c. greater at 15° C. than at 20° C.

The weight of tobacco mosaic virus required per lesion produced as given above represents the average for a batch of 64 half-leaves, and much lower values are obtained by considering a single susceptible leaf. For example, in another experiment 6×10^{-8} gm. virus in 0·1 ml. inoculum produced 53 lesions, giving an average infective unit of $1{\cdot}1 \times 10^{-9}$ gm. per lesion for this half-leaf.

On the basis of the precipitin titre of infective tobacco mosaic juice against immune serum, and on the assumption that the virus is antigenically comparable with egg albumen, Chester (1935) has estimated the weight of virus in 1 ml. of infective juice to be about 1 milligram. With a further assumption of 100,000 for the molecular weight of the virus particle (Robbins, 1934), he has concluded that a "minimum infective dose" of 6×10^{-8} particles is required for the production of a single lesion.

In the absence of an experimentally determined value for the molecular weight of the mosaic particle, the number of particles calculated to be contained in a given weight will depend on the value assumed for the molecular weight, but a reasonable estimate of this may be made from a knowledge of the size of the particle and its nature. The most reliable experiments recorded give values for the diameter of the particle ranging from $5{\cdot}5\mu\mu$ to $15\mu\mu$. It also appears from the work of Stanley (1935, 1936), and that of the author (1936a), that this virus is protein in nature, so that by analogy with other proteins we may estimate the molecular weight at about 5×10^5, with reasonable expectation that it will not be less than one-tenth or more than ten times this value. On this basis the average infective unit of $1{\cdot}1 \times 10^{-9}$ gm. virus given above would represent $1{\cdot}3 \times 10^9$ virus particles.

Finally, the bearing of the results on the use of the local lesion method of estimating relative virus concentrations is fairly obvious. On account of the form of the lesion appearance curves, it is clear that normally counts should be made when the limiting number is being approached. In the case of T.S.W. there is the further circumstance that lesions produced by dilute inocula increase proportionately more at later counts than do those produced by more concentrated inocula. This aspect is so much bound up with the form of the dilution curves that a fuller consideration will be given in a later paper dealing with dilution curves in general.

SUMMARY.

Some effects of light and temperature on the rate of appearance and the total number of primary lesions of the viruses of tomato spotted wilt and tobacco mosaic on *N. tabacum* and *N. glutinosa* respectively, have been investigated.

Under constant conditions of light and temperature, the curves relating the number of lesions to time were found to be sigmoid in form, and to approach limiting values.

The limiting value was 30 p.c. higher at 20° C. than at 15° C. for both virus-host combinations when the light factor was kept constant.

The time interval between inoculation and the time of maximum rate of appearance of lesions varies with temperature, and at constant temperature appears to be a constant for any one virus-host combination. This time interval was found to be 50 p.c. longer at 15° C. than at 20° C. for mosaic lesions on *N. glutinosa*, and 60 p.c. longer for T.S.W. lesions on *N. tabacum*.

The time taken by T.S.W. lesions to attain their maximum rate of appearance was 60 p.c. longer than that for mosaic lesions at 20° C., and 50 p.c. longer at 15° C.

Neither sunlight nor artificial light appeared to affect tobacco mosaic virus lesions quantitatively.

Sunlight was found to exert an inhibiting action on the appearance of tomato spotted wilt lesions on *N. tabacum*. A similar but much feebler effect was observed with artificial light.

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INFLUENZA ON THE DEVELOPING EGG[1]

2. TITRATION OF EGG PASSAGE VIRUS BY THE POCK-COUNTING METHOD

by

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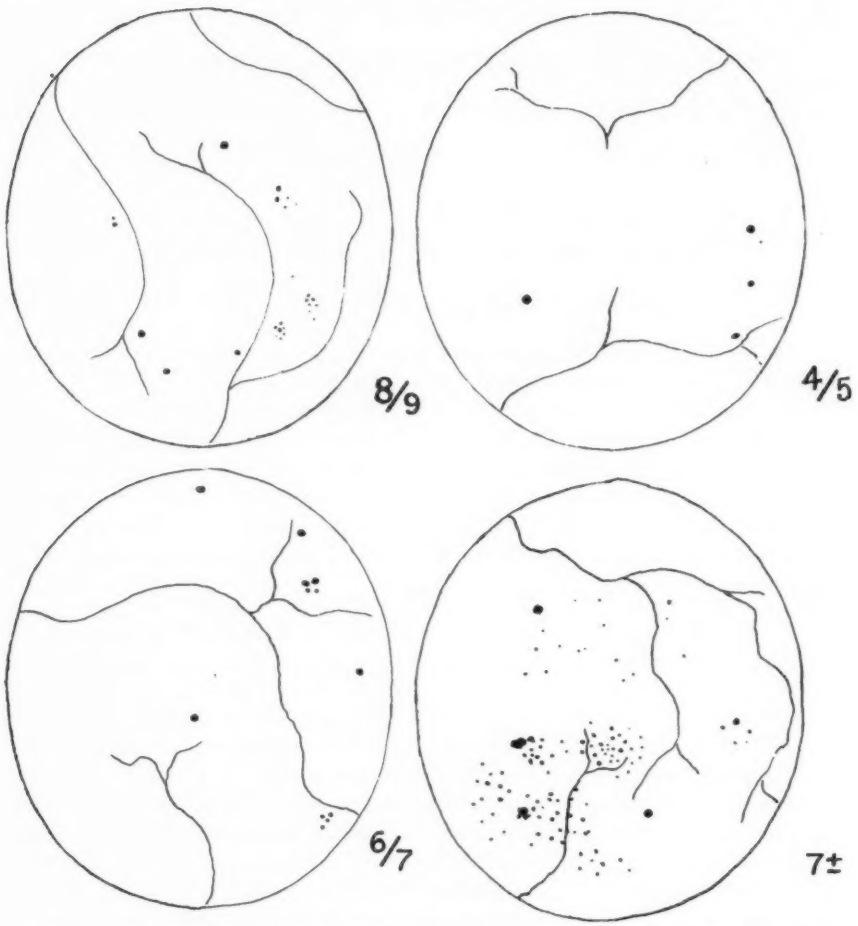
(Submitted for publication 7th August, 1936.)

As soon as the egg passage strain of influenza virus (Burnet, 1936b) was producing easily recognizable lesions on the chorioallantoic membrane, attempts were made to titrate the virus by the "pock counting" method, which has been successfully used for other viruses such as laryngotracheitis of fowls (Burnet, 1936a) and vaccinia (Keogh, 1936). Certain difficulties were encountered which will be discussed below, but with the well-established virus very satisfactory results are obtainable. It can probably be claimed that, excluding the bacteriophages, egg passage influenza virus can be titrated with greater accuracy than any other virus.

Characteristics of the Focal Lesions—Satellitism.

For all experiments the stock virus suspension is prepared by grinding a freshly-removed membrane showing semi-confluent lesions with sterile quartz powder and 5 c.c. of nutrient broth. The finely-ground emulsion is centrifuged for ten minutes in a small angle centrifuge, and the slightly opalescent supernatant fluid carefully removed. If serial tenfold dilutions of this stock suspension are inoculated into eggs according to the standard technique, it will be found that almost without exception the eggs inoculated with a 1 : 1,000 dilution will show a conveniently small number of focal lesions between 5 and 30. Out of a large number of tests with such 1 : 1,000 dilutions, slightly under 50 per cent. gave easily counted lesions in the form of single well separated foci. The others showed in varying degree the phenomenon of secondary foci or satellitism. This has been described in a previous paper (Burnet, 1936b). Instead of a single focal lesion, one may have either a relatively large central focus with small secondary ones scattered irregularly around it, or a group of small foci without any distinct parent lesion. When there are very small numbers of primary foci present, it is easy to recognize the nature of these secondary satellite systems, but when numbers become larger, fusion of the different systems often makes it impossible to recognize primary from secondary foci, and no count is possible. Figure 1 shows four diagrams of the distribution of foci on selected membranes, which may make the nature of the difficulty clearer.

[1] The first paper of this series appeared in the British Journal of Experimental Pathology.



Diagrams of the distribution of specific foci on four membranes from an influenza virus titration to illustrate the difference between primary and secondary foci. The count recorded from each membrane is shown alongside.

In counting the lesions in influenza virus titration experiments, a certain arbitrariness of interpretation is unavoidable. It is impossible to adopt any fixed objective standard as to what is to be regarded as a primary focus. Some membranes will show obvious secondary foci which are much larger than equally obvious primary foci on another membrane. The best guide as to the primary or secondary nature of foci is to be gained from their distribution. Discrete foci

fairly uniformly distributed over the whole area on which any foci occur may safely be regarded as primary foci. Where a patchy distribution indicates that a large number of secondary foci are present, the primary foci are usually larger, and show a more distinct mesodermal haze surrounding them. In practice one finds that when a number of membranes from eggs receiving the same inoculum are examined, counts which agree closely are obtained if one proceeds on the basis of determining the *smallest* number of primary foci which could reasonably account for the observed distribution. To minimize as far as possible the personal bias in favour of "good duplicates", and to make the results as objective as possible, a second count is made to include those foci about which there is a reasonable doubt as to whether they are primary or secondary. In detailed protocols the counts of foci are given in the form a/b, "a" being the minimum, and "b" the maximum count of primary foci. It is not infrequent to find a membrane showing a large number (i.e. over 25) of foci which are clearly primary, but with too many well-developed secondaries to allow a count of all the "possible" primary lesions. Under these circumstances, a careful estimate of the number of undoubted primary foci is made, and the result recorded as a $\pm$ instead of a/b. In averaging and interpreting the results, only the minimum counts (a) are used.

The Relation between Virus Concentration and Pock Counts.

As far as can be determined over the limited range available, the number of foci produced is directly proportional to the virus concentration. Table 1 shows the counts obtained when a stock virus suspension was titrated in doubling dilutions from 1 : 500 to 1 : 4,000.

TABLE 1.

Linear Relationship of Pock Count to Virus Concentration.

Virus Dilution.	Pock Counts.					Average.
1: 500	32/36	25/37	+	+	+	28·5
1: 1000	17/19	18/24	+	+	+	17·5
1: 2000	8/9	9/15	10/12	8/16	+	8·75
1: 4000	2/3	6/8	4/5	6/9	+	4·5

+ Count impossible owing to excessive satellite formation.

The degree of satellite formation in this batch of eggs was distinctly greater than usual : it is very rare to find any completely uncountable membranes when the average count is 10 or under. One feature of this table calls for immediate comment—the absence of unduly low counts. With all the other viruses which we have studied, there has been a proportion of membranes giving counts as low as 10 p.c. of the average count obtained with the inoculum in question. The counts with egg-adapted influenza virus rarely fall outside the range to be expected from random variation and minor technical inaccuracies. Occasionally one observes a membrane which shows very small foci whose number may be significantly low, but

these are rare. It is clear that the virus has a high virulence for the egg membrane, and that eggs vary little in their susceptibility under the conditions of incubation.

As described in the first paper of this series, the virus, after about 70 successive passages, is invariably fatal for the embryo in three days, producing easily recognized lesions in the form of multiple haemorrhages, sometimes involving numerous tissues, but more usually confined to the brain. This allows a comparison to be made between the pock-producing capacity of the virus and the limit to which the lethal effect may be obtained.

In the experiment, whose results are shown in Table 2, four eggs were inoculated with stock virus diluted 1 : 1,000, four with 1 : 2,000, and nine with 1 : 20,000, the dilutions being made in saline containing 10 p.c. normal horse serum. The first two series were opened at two days, and the specific foci counted. The eggs containing virus diluted 1 : 20,000 were incubated for three days and then examined by transillumination. Four were certainly dead, two appeared doubtful, while three contained active embryos. The dead eggs were opened and found to contain embryos with typical haemorrhagic encephalitis. On the following day (four days after inoculation), the remaining five eggs were opened. Two were dead with typical lesions, three alive. Two of the latter, however, showed small foci, probably blood borne, on the membranes, and subinoculations from the ground-up membranes produced typical egg lesions. The remaining egg showed no lesions, and the membrane proved non-infective.

TABLE 2.

Comparison of Pock Counts with Lethal Action of Virus.

Virus Dilution.	Result.									
1 : 1000	9/10		13/18		8/10		11/12		(10)	
1 : 2000	4/12		2/2		4/4		5/9		(4)	
1 : 20000	†3	†3	†3	†3	†4	†4	S+	S+	S—	(0·89)

†3. Embryo dead at three days.
S. Embryo alive at four days.
+. Virus present.
—. No virus present.

From these results it is evident that the pock count does not represent the full number of virus particles inoculated on to the egg membrane. About half appear to be capable of entering the embryo without provoking a membrane lesion. This is not surprising when one considers the extensive plexus of thin-walled capillaries lying on the surface of the membrane, and the fact, previously established, that the virus may be carried by blood cells.

A similar experiment carried out in duplicate at temperatures of 36·5° C. (the standard temperature of incubation used for experiments with influenza virus) and 39° C. gave some interesting results, which are tabulated in Table 3.

TABLE 3.

Comparison of Pock Counts and Lethal Action at 36·5° C. and 39° C.

Virus Dilution.	Temperature of Incubation.	Result.					Average.
1 :2000	36·5°	3/3	2/2	4/7	2/2	5/6	3·2
	39°	1/1	4/4	0/1	2/2	+	1·75
1: 20000	36·5°	†	†	†	†	†	†
	39°	S	S	S	S	S	S

† Embryo dead at five days. S Embryo alive at five days.

It will be seen that the pock count is significantly lower at 39° C. The foci were also distinctly smaller than those obtained at lower temperatures. The eggs containing 1 : 20,000 dilution of virus were opened at five days, and two of the deaths may have been non-specific, since the typical haemorrhages were absent, but it is quite clear that at 39° C. there is a much more effective resistance to infection by very small numbers of virus particles. A few experiments with larger amounts of virus in eggs incubated at 39° C. gave death at three days with haemorrhagic encephalitis.

DISCUSSION.

These results are in most respects what might be expected of a virus whose pathogenicity for the ectodermal cells of the chorioallantois, and probably also for many other types of embryonic cell, has been brought to a high level by passage. The number of primary foci produced is certainly smaller than the number of viable virus particles introduced into the egg. It is impossible to assess actually what proportion of virus particles does produce foci, as we have no means of ascertaining with what regularity a single particle can induce infection. It has been shown that an amount of virus which killed eggs incubated at 36·5° C. was innocuous when the eggs were incubated at 39° C. The embryo tissues, therefore (at least at the higher temperature), have some power of rendering small numbers of virus particles inactive, and to a lesser extent this may be true at 36·5° C. also. All that can be deduced from the linear relationship between concentration of virus and pock counts is that each primary focus is initiated by a single virus particle, and that an approximately constant proportion of the virus particles inoculated gives rise to specific foci. The appearance of secondary foci is presumably due to transport of virus particles produced in the primary lesion, either by simple diffusion or in leucocytes. The proof that the number of foci is not a direct indication of the total number of viable particles does not in any way invalidate the titration results. Analogous relationships are found with bacterial plate counts, and in bacteriophage titrations by plaque counting methods. Except in special circumstances, it is probably legitimate to discuss the results of experimental titrations as if the foci did represent the total number of viable particles.

SUMMARY.

Egg passage influenza virus can be titrated by "pock counting" methods on the chorioallantois, provided care is taken to distinguish primary from secondary foci.

The number of foci produced is less than the number of viable virus particles introduced, as judged by comparison of pock counts with the lethal effect on the embryo.

Higher counts of foci and a smaller minimum lethal dose are obtained with eggs incubated at 36·5° C. than at 39° C.

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INFLUENZA ON THE DEVELOPING EGG

3. THE "NEUTRALIZATION" OF EGG VIRUS BY IMMUNE SERA

by

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One of the chief uses of the egg membrane technique is for the demonstration and quantitative estimation of specific antibody in sera from animals which have been immunized with viruses which can be titrated by this method. The first application of this sort was made by Burnet and Galloway (1934), who showed that the two serologically distinct strains of vesicular stomatitis virus could be rendered non-infective for the chick embryo by the homologous immune serum and not by the heterologous serum. Higbie and Howitt (1935) obtained equally satisfactory results with the Eastern and Western strains of equine encephalomyelitis. Pock counting methods which were not applicable to these viruses, were applied to serological work with the virus of laryngotracheitis of fowls by Burnet (1936a). Other viruses which can be studied in the same way include vaccinia (Keogh, 1936), infectious ectromelia of mice (Burnet and Lush, 1936a), and the avian pox viruses (Burnet and Lush, 1936b).

The virus of epidemic influenza can be shown to be readily inactivated by immune sera from man or animals by tests on ferrets (Smith, Andrewes, and Laidlaw, 1933), or on mice (Andrewes, Laidlaw, and Smith, 1934). Specific inactivation of egg passage virus by immune sera can also be readily demonstrated on the chorioallantoic membrane. A brief account of the influence of ferret-immune sera on egg virus has already been published (Burnet, 1936b). With one serum a mixture of undiluted virus and serum diluted 1 : 100 gave a count of foci about 0·1 p.c. of the corresponding control count. During the last six months this work has been extended; a large number of immune sera from man, horse, ferret, and mice have been tested against egg virus, and the results compared with those of mouse tests made according to the Hampstead technique. The reactions between egg virus and high titre ferret serum have been studied in detail, with a view toward providing relevant data to help in the elucidation of the still controversial problems of the nature of the virus-immune serum interactions. This paper is concerned primarily with the reaction between immune ferret sera and egg passage virus, results obtained with other sera being introduced only to indicate the general applicability of the results obtained.

METHODS.

Fully adapted egg virus in its 65th to 72nd egg passages was used in all the experiments described. The stock virus emulsion was prepared freshly for each experiment from a membrane showing typical lesions either freshly removed or stored in broth for not more than 48 hours in the refrigerator. Each membrane was ground in a mortar with sterile quartz powder and emulsified in 5 ml. of broth. This emulsion was spun for ten minutes in a small angle centrifuge at 3,500 r.p.m., and the supernatant fluid used as stock virus.

The serum chiefly used, F.22, was obtained from a ferret ten days after being infected with mouse passage virus, and showing mild symptoms. It was stored unfiltered for some months, but was passed through a Seitz filter before the present experiments were commenced, owing to the appearance of mould. Serum F.38 was also from a ferret infected with mouse passage virus eleven days previously, and showing typical symptoms.

Suitable dilutions of serum and virus were made either in Ca-saline (normal saline to which the Ringer proportion of $CaCl_2$ had been added) or in 10 p.c. normal horse serum saline (N.H.S. saline). The latter diluent was used for all later experiments since it allowed very dilute virus to retain its activity better than in the simple saline, but no difference between the two diluents could be observed in the usual serum-virus mixture tests. All the published protocols with the exception of that given in Table 1, are of experiments in which N.H.S. saline was used as diluent.

Serum-virus mixtures were always made by adding an equal volume of serum suitably diluted to the virus dilution. The final dilutions of the reagents in the mixtures are throughout twice that shown in the protocols. For "immediate" tests, the tube was thoroughly shaken for about five seconds, and then either two or three 0·05 ml. quantities inoculated into previously opened eggs. The mixtures were kept at room temperature in the dark, or if on the bench during inoculations, shielded from direct daylight.

The methods of egg inoculation have been fully described elsewhere (Burnett and Galloway, 1934), and no modification was introduced in the present work. The characteristics of influenza virus foci and the difficulties in their enumeration are discussed in the preceding paper.

Incomplete Reproducibility of Results.

It will be recognized that an immunological technique in which the action of one living organism on another is used as the indicator will be subject to many variables which may not be easy to control. In the course of work with the present reaction, it has been found that while the results of any one experiment done on the same day with a single batch of eggs and the same stock virus suspension are internally consistent, experiments done on different occasions may show very considerable differences in the degree to which the virus is inactivated by a constant serum dilution.

A series of experiments under approximately similar conditions, in which serum F.22 diluted 1 : 100 was added to an equal volume of undiluted stock virus and titrated after one to two hours at room temperature, gave the following percentages of survivors as judged by pock counts, the order being that in which the experiments were carried out over a period of four months:

0·04%, 0·08%, 0·05%, 0·05%, 0·10%, 0·14%, 0·11%, 0·05%, 0·02%, 0·60%, 0·06%, 0·16%, 0·14%.

While most of the results lie between 0·05% and 0·14%, two very discrepant results, 0·02% 0·60%, are included. With 1 : 50 serum three experiments gave one

discrepant result, 0·02%, 0·028%, 0·17%. The last unduly high figure is derived from the experiment whose protocol is given in Table 5.

The earlier experiments were made with serum diluted in saline; for the latter ones 10 p.c. normal horse serum saline was used as diluent. Direct comparison on the same day showed no significant difference between the results, apart from better survival of the control virus in the diluent containing normal serum.

There appear to be three main reasons for this variability, though other unrecognized factors also may be involved. In the first place the determination of the percentage of survivors necessarily requires a knowledge of the number of foci produced in the absence of serum. Like most active biological materials, influenza virus retains its activity much better in the form of an undiluted stock suspension containing a good deal of tissue protein, etc., than when highly diluted in any simpler solution. The control titrations were almost always made with 1 : 1,000 dilutions of stock virus, and it is likely that even when the diluent was N.H.S. saline the highly dilute virus would be more susceptible to minor uncontrolled factors such as exposure to light and delay between making the dilution and titrating.

The second source of variation in the results is to be found in the variability of the extent to which the secondary irreversible phase of the interaction between virus and antiserum occurs. This is illustrated and discussed in later sections.

Finally, it has been found that there are differences of unknown origin between different stock virus suspensions in regard to their behaviour with serum. An experiment was done to compare the results when two different egg virus suspensions were used. Each of these was probably derived within a few generations from a single virus particle. The strain, S.P.1, was obtained from a membrane showing only two well developed foci situated far apart. The membrane surface was dry, and a small droplet of broth was placed over one lesion and then recovered with a capillary pipette. This drop inoculated to another egg gave only three foci. This second membrane served as the source of S.P.1, the strain used in most of the experiments whose protocols are given. The second strain, S.P.2, was derived from an egg inoculated with the limiting dilution of 1 : 20,000, in which the embryo was still alive at the fourth day. The two virus emulsions were made as usual, and the conditions of experiment identical throughout, two ferret sera, F.22 and F.38, being used with each strain of virus. The results given in Table 1 show a significant difference in the case of inactivation of the two strains.

TABLE 1.

Difference in Reactions of Two "Single Pock" Strains of Egg Virus.

Virus.	Serum.	Final Serum Dilution. 20.			200.				Equivalent Control.
S.P.1	F.22	0	1/1	(0·5)	9/10	12/15	1/3	(7)	12,000
S.P.2	„	4/4	3/3	(3·5)	13 +	20 +	7/7	(13)	8,000
S.P.1	F.38	0	0	(0)	2/2	2/2	4/6	(3)	12,000
S.P.2	„	1/1	6/7	(3·5)	7/9	9/10	8/8	(8)	8,000

The increased resistance of S.P.2 to inactivation by serum diluted 1:20 is particularly noteworthy.

Differences in substrain used for the experiment, however, cannot be regarded as responsible for all the variations observed, since the two most discordant results

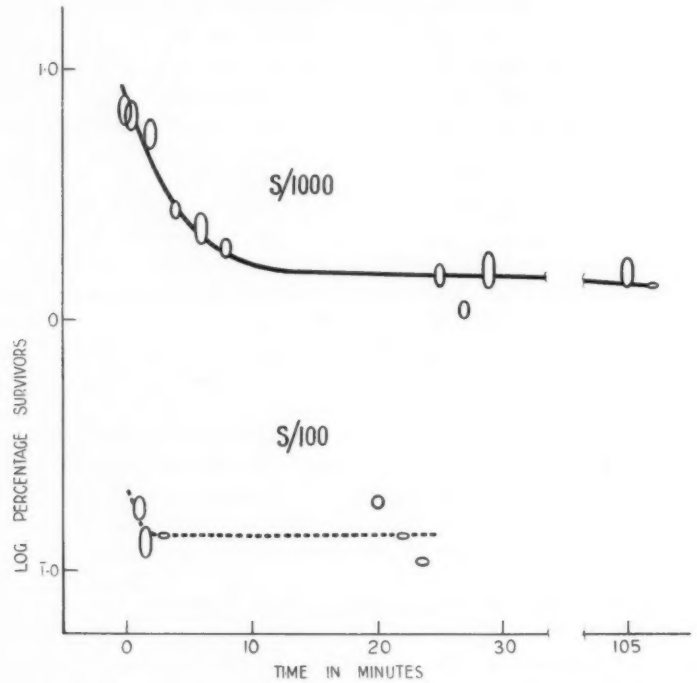

Fig. 1. The influence of time of contact on the inactivation of virus by immune serum. Log. percentage "survivors" plotted against time in minutes elapsing between mixing reagents and testing by egg inoculation. Each oval represents the count on one egg membrane, the height of the oval giving a measure of the possible error in determining the count of primary foci. Continuous line, virus 1 : 10 with serum F.38 1 : 1,000, broken line, undiluted virus with serum 1 : 100.

were obtained with the same S.P.1 strain. An attempt to determine whether such differences might be due to varying amounts of blood, allantoic fluid, etc., removed with the membranes, gave a completely negative result, and the discrepancies must at present be left without a satisfactory explanation.

The Influence of Time on the Reaction between Virus and Antiserum.

A large number of experiments has been made to determine the influence of time of contact on the result obtained when virus-immune serum mixtures were

inoculated on to egg membranes. There was no difficulty in showing that when inoculations were made within 20 seconds of mixing, the counts obtained were always higher than those obtained with the same mixtures after they had stood for one to one-and-a-half hours at room temperature. The differences obtained, however, varied greatly from experiment to experiment, and the interpretation of the results was not clear until evidence from other sources indicated that in all probability two processes were taking place, a rapid approach to an equilibrium, and then a slow further loss of activity. Fig. 1 shows the results obtained when virus undiluted was mixed with serum F.38 (1 : 100) and virus diluted 1 : 10 with serum diluted 1 : 1,000, egg inoculations being made at intervals from 20 seconds to one-and-a-half hours.

This figure shows the salient features of the reaction very clearly. With relatively strong serum (1 : 100), the approximate equilibrium level is reached within a minute or two. With serum diluted 1 : 1,000 there is a distinct progressive reduction in the count over the first ten minutes, but an equilibrium level is reached in 15-20 minutes, which falls only slightly with an hour's further contact.

All the experiments which have been made along these lines have given results consistent with this interpretation. The rapid approach to an equilibrium state is constantly observed, but the degree to which the (apparently secondary) further fall proceeds has varied considerably from experiment to experiment. Three examples may be given.

Experiment 1. Undiluted stock virus was mixed with 1 :50 dilution of serum F.22, both components having been previously chilled and the mixture maintained for the first fifteen minutes in an ice water bath. Eggs previously opened were inoculated as rapidly as possible after the mixture had been thoroughly shaken, and again at fifteen minutes. The mixture was then placed at room temperature in the dark for two hours, and again inoculated to three eggs. The control was a mixture of 1: 50 normal serum and undiluted virus. This was maintained under the same conditions as the experimental mixture and titrated before and after the experiment by making 1: 1000 dilution in cold saline and testing this.

TABLE 2.

Influence of Time on Virus Inactivation by Immune Serum.

		Time of Testing.								
		Immediately.			15 min. at 0°C.			2 hours at R.T.		
F.22	1: 50 + Undiluted Virus	+	+	3	4	1	2	3	2	0
N.S.	1: 50 + Undiluted Virus (diluted 1: 1000)		6	10					+	12

This was an early experiment, and no attempt was made to count the membranes showing numerous secondary foci, as was done in later experiments, but the immediate counts marked "+" undoubtedly represented considerably more than three foci. The last of the "immediate" series was inoculated about one minute after mixing, and has apparently already reached the approximate equilibrium value of 3. Very little change takes place thereafter.

Another experiment with the same reagents, done some months later, is included in Table 5 as part of a larger experiment to be discussed in connection with dissociation of the virus-antivirus union. In this there is no significant difference between the results obtained 1–2 minutes and 20 minutes after mixing, but a further fall to about 60 p.c. of this value after 65–70 minutes. Table 4 shows another example in which the secondary fall with 1 : 100 serum is unusually marked.

The "Percentage Law".

Andrewes and Elford's (1933) discovery that the percentage reduction of phage titre by antiserum is dependent only on the antibody concentration and independent of the phage concentration has been shown to be equally applicable to the neutralization of laryngotracheitis (Burnet, 1936a) and vaccinia viruses (Keogh, 1936), as demonstrated on the egg membrane. With influenza virus inactivation, the "percentage law" also holds. Tests over an extensive range of concentrations are impracticable, owing to the difficulty of counting large numbers of foci and the inadmissibility of making any further dilution of the serum-virus mixture, on account of the dissociation phenomena described in a later section. The whole series of neutralization experiments, however, left the impression that the percentage law probably held over the whole range. Table 3 shows the result of an experiment to test its applicability over a limited range. Virus undiluted and diluted 1 : 2, 1 : 5, and 1 : 10 was mixed with serum F.22 diluted 1 : 100. After the mixtures had stood for twelve minutes at room temperature, egg inoculations were commenced and completed 17 minutes later.

TABLE 3.

The Percentage Law with Egg Virus Neutralizations.

Mixture.	Focal Counts.			Average.
Serum 1 : 100 + Undiluted Virus	108 ±	128 ±	+	118
„ + Virus 1 : 2	66/77	69 ±	+	68
„ + „ 1 : 5	23/25	27/32	32/36	27
„ + „ 1 : 10	21/31	5/6	14/15	13

The higher counts shown in this table are subject to considerable error, but they were made before the eggs showing the lower counts were opened, and were therefore uninfluenced by any unconscious tendency to obtain the "right" count.

The same type of result has been obtained incidentally in many experiments, and throughout this work the percentage law has been tacitly accepted as valid in designing experiments and in interpreting the results.

The Relationship between Serum Concentration and Percentage of Survivors.

As in all virus and phage neutralization reactions, the more concentrated the antiserum the smaller is the amount of virus which survives. In the case of influenza virus neutralization, the proportion of foci produced is, within the limits of

experimental error, inversely proportional to the serum concentration. If, for example, 1 : 100 immune serum added to undiluted virus gives 0·1 p.c. of the foci obtained with normal serum, 1 : 1,000 serum will give 1 p.c., and so on.

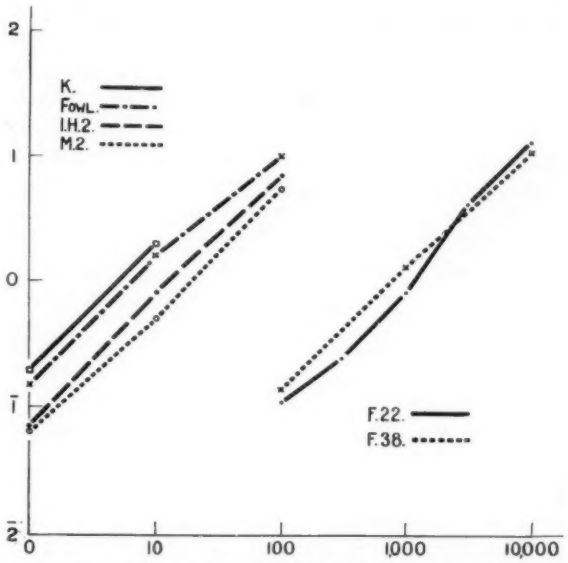

Fig. 2. To show relation between serum dilution and percentage of "survivors". Log. percentage plotted against log. serum dilution. Two ferret sera, F.22, F.38, and one each of horse (I.H.2), mouse (M.2), fowl (F.29), and human (K) origin are shown.

Figure 2 shows the relationship between serum concentration and proportion of survivors for two immune ferret sera, and one serum each of human, horse, fowl, and mouse origin. The two variables are plotted logarithmically, and in each case the points lie approximately on a straight line running at an angle of 45°. Since the undiluted virus gives, in nearly all instances, a count approximating 10,000, it is not possible to obtain countable numbers below 0·1 p.c., and when there are more than 10 p.c. of survivors the results become very irregular, so that only a hundredfold range of serum concentration is available for test.

In a large number of experiments it has been found that when a serum, diluted so as to give with undiluted virus about 0·1 p.c. of survivors, is tested diluted against virus diluted 1 : 10, the same number of survivors (i.e. 1 p.c.) is obtained when the period of contact is one to two hours. When both reagents are further diluted tenfold, it has been usual to obtain approximately the same count, but significant deviations from it are more frequent.

On a few occasions results have been obtained in which higher dilutions of

serum have had a relatively greater inactivating effect. An example is shown in the action of both ferret sera on the strain S.P.2 in Table 1, and one test with a moderately active human serum gave a very similar result. The great majority of experiments, however, have shown the typical relationship between serum dilution and percentage of survivors, and the significance of these exceptions has not yet been studied.

The relationship between serum concentration C. and the percentage of survivors P. (C.P. = constant) holds even when considerably different absolute degrees of inactivation are obtained in different experiments with the same serum. Thus on the occasion when F.22, diluted 1 : 100, gave 0·6 p.c. survivors, 1 : 1,000 serum gave 7·0 p.c., and 1 : 10,000 no appreciable reduction as compared with the plotted results 0·11 p.c., 0·8 p.c., and 13 p.c. Similarly, serum F.38 gave with 1 : 100 and 1 : 1,000 serum 0·075 p.c. and 0·7 p.c. survivors on one occasion, 0·18 p.c. and 2·1 p.c. on another, and 0·14 p.c. and 1·3 p.c. on a third test.

Dissociation of Virus-Antiserum Mixtures on Dilution.

It has been shown for several different viruses that when a mixture, containing just sufficient antiserum to render the virus non-infective, is diluted with saline or other inert fluid, the diluted mixture is again infective. If the original neutralization was due to virus-antibody union, the union is readily dissociable by simple dilution. The same phenomenon can be observed with influenza virus inactivations. We may take a mixture of serum and virus which has been long enough in contact to reach the approximate equilibrium level described earlier, and on titrating produces x foci. If this is diluted 1 : 10 in saline and titrated after a few minutes, the pock counts obtained will lie between two possible limits. If no dissociation occurs, the count will be x/10; if complete dissociation occurs, the count will be the same as is obtained by mixing virus and serum, each initially diluted 1 : 10. According to the "C.P. = constant" rule, this number will be x, so that if complete dissociation occurs, dilution of a mixture should produce no change in the number of foci developing on titration.

Experiment shows that to a good approximation complete dissociation takes place if the dilution is made fairly soon after mixing. With longer contact, the degree of dissociation is decreased, apparently in proportion to the progress of the second phase of the inactivation.

Protocols of two experiments will be given to illustrate the conditions under which dissociation occurs, and its extent.

Experiment 2. The following mixtures of virus and serum F.22 were set up, all dilutions being in N.H.S. saline, (1) V + S/100, (2) V/10 + S/1,000, (3) V/100 + S/10,000. Mixture 1 was titrated within one minute after mixing, and again a little over an hour later. As soon as the first titrations had been made, portion of the mixture was diluted 1: 10 and 1: 100 in N.H.S. saline. These dilutions were titrated after standing 60–80 minutes at room temperature. Half an hour after making the original mixture 1, a second series of 1: 10 and 1: 100 dilutions were made from it. These were titrated twice, immediately and after 30–40 minutes. Original mixtures 2 and 3 were titrated a little over an hour after mixing. Table 4 combines the results.

TABLE 4.

Dissociation of Virus-Antibody Union on Dilution.

Approximate Time of Testing.	V + S/100.	Diluted at Once. 1 : 10	Diluted at Once. 1 : 100	Diluted at 30 Minutes. 1 : 10	Diluted at 30 Minutes. 1 : 100	V/10 + S/1,000	V/100 + S/10,000
Immediately	10/11 12/23 (11)						
30–40 minutes				4/4 3/3 2/3 (3)	0/1 1/1 0 (0·3)		
60–90 minutes	0 1/2 1/1 (0·7)	8/12 9/9 (8·5)	9/9 8/15 (8·5)	3/7 1/1 1/2 (1·7)	1/1 1/2 2/3 (1·3)	6/9 11/12 (8·5)	14/17 5/6 (9·5)

Original virus titre, V/2 = 4,500.

In summary, this experiment shows that, immediately after mixing, the survivor count has been reduced from 4,500 to 11. Immediate dilution of this mixture 1 : 10 and 1 : 100 gives an almost equal count in each case, this count being the same as that obtained if similar dilute mixtures are reached by mixing the previously diluted components.

After an hour, the proportion of survivors has been reduced to an unusually low level (0·7). Dilutions made from the mixture at 30 minutes do not show the full dissociation of the earlier dilutions. The figures are small, and it is doubtful if the differences between the two sets of titrations are significant. It is probably better to combine them, when the average count of foci will be for the 1 : 10 dilution 2·3; for the 1 : 100, 1·0. This indicates that while the process responsible for the immediate fall on mixing the components is reversible, the subsequent inactivation involves, in part at least, a process which is not readily reversible by simple dilution.

Experiment 3. This experiment was carried out along very similar lines, but the original mixture was made with serum F.22 diluted 1 : 50, and only 1 : 10 dilutions of the mixture were titrated. The original mixture was titrated 1 minute, 20 minutes, and around 70 minutes after mixing, while 1 : 10 dilutions made from this at 20 minutes and at one hour were each titrated immediately upon dilution, and half an hour later.

TABLE 5.

Dissociation of Serum-Virus Mixture by Dilution.

Mixtures.	Times of testing after primary mixture. 1 minute.	20 minutes.	60–70 minutes.	80–90 minutes.
V + S/50	33/36 35/37 (34)	33/43 38/40 49 ± (40)	19/22 15/15 25/31 (20)	
Diluted 1 : 10 at 20 minutes		34 ± 29/40 22/22 (28)	31/33 24/26 29 ± (28)	
Diluted 1 : 10 at 60 minutes			15/18 11/12 13/15 (13)	7/8 14/17 (11)

The counts in Table 5 are relatively much higher than in the first experiment, i.e. the inactivation is less complete, but the general character of the results is identical. There is no significant difference between the 1 minute and 20 minute counts, but after an hour there has been a significant fall. The tenfold dilution of the mixture made at 20 minutes gives a count which is approximately 80 p.c. of that given by the original mixture. This remains unchanged. Dilution after an hour's contact gives definitely lower values, indicating again that a slow irreversible change follows the rapid initial equilibration.

The same almost complete dissociation is observable with human-immune sera. Table 6 shows the results of an experiment in which human serum K. was mixed undiluted with stock virus. After 30 minutes at room temperature, portion of the mixture was diluted 1 : 10 in N.H.S. saline. From 1–1½ hours after making the original mixtures, titrations were made (a) of the original mixture, (b) of the 1 : 10 dilution therefrom, and (c) of a mixture of serum and virus each diluted 1 : 10 before mixing.

TABLE 6.

Dissociation of Virus-Human Immune Serum Mixture.

Material.	Pock Counts.				Percentage "survivors".
Serum K. + Virus	12/20	17/25	10/12	(13)	0·22%
Do., diluted 1: 10 at 30 minutes	8/10	9/9	8/9	(8)	1·3%
Serum 1: 10 + Virus 1: 10	14/14	14/16	10 ±	(13)	2·2%
Virus 1: 1,000	11/12	9/12	14/21	(11)	

This repeats the chief features of the other two experiments, and shows the typical relationship between the two serum concentrations and a high degree of dissociation on dilution, not, however, complete, when the original mixture had stood for 30 minutes before dilution.

DISCUSSION.

These results establish certain points in regard to the influenza virus-antiserum reaction, and inferentially, in regard to virus-antibody reactions in general. In view of the controversy as to whether any union between virus and antibody takes place during the early stages of the reaction, the result expressed in Fig. 1 is, perhaps, the most important. With dilute serum, a certain time must elapse before the characteristic equilibrium level is attained; with stronger serum an equilibrium is reached almost immediately after mixing. This is direct evidence that a reaction, whose velocity is proportional to the concentration of serum present, takes place between virus and antibody. From general immunological experience, this reaction can only be a union between antigen on the surface of the virus particles and antibody.

At least during the early stages this union has been shown to be almost completely reversible, so that the equilibria should conform to the ordinary mass action laws. If certain reasonable assumptions are made concerning the nature of the virus antibody reaction, it can be readily deduced that under standardized conditions $C.P.$ is a constant, where $C.$ is the concentration of antibody, and $P.$ the proportion of survivors capable of producing focal lesions.

The simplest possible assumption in regard to the reaction is that union between a virus particle and an antibody molecule results in the production of a non-infective compound. If the initial concentration of virus is $N.$ particles per unit volume, the number of "survivors" at equilibrium, n, and the initial concentration of antibody, $C.$, then at equilibrium the concentration of the three components will be:

$$\begin{array}{ccccc} \text{Virus} & + & \text{antibody} & \rightleftharpoons & \text{non-infective compound} \\ n & & C-(N-n) & & N-n \end{array}$$

$$\text{By mass action law, } \frac{n\,[C-(N-n)]}{N-n} = \text{constant.}$$

By analogy with the results obtained with vaccinia virus (Andrewes, 1928; Sabin, 1933), and with bacteriophages (Andrewes and Elford, 1933), one can be certain that a great excess of antibody is present, so that $[C-(N-n)]$ will practically equal $C.$ $\therefore \frac{n}{N-n} \cdot C = \text{constant.}$ When n is small compared to $N.$, $\frac{n}{N}$ (i.e. the proportion of survivors, $P.$) $\times\, C = \text{constant.}$

Similar reasoning will hold if, instead of a single union between virus particle and antibody molecule, we postulate what, on biological grounds, is far more probable, viz. that each virus particle carries $p.$ antigenic groups, each capable of reacting with a single antibody molecule, and that $q.$ of these groups must be free of antibody if the virus particle is to manifest its activity. Under these conditions, and on the assumption that when only a small proportion of survivors remains, they may all be regarded as having only $q.$ active groups uncombined, the equilibrium equation becomes $\frac{an[C-(pN-qn)]}{pN-qn} = \text{constant.}$ Making the same assumptions as previously, $C \cdot q/p \cdot \frac{n}{N} = \text{constant}$, i.e. $C.P. = \text{constant}$, as before.

Experimentally, it has been shown that for a given concentration of serum, $P.$ is independent of the amount of virus (the percentage law), and that when serum concentration is varied, the product $C.P.$ is nearly constant with immune sera from several animal species. This concordance with theory appears to indicate that the virus particles must be of homogeneous character, the number of antibody molecules necessary to inactivate being similar for all particles.

This reasoning concerns only the primary, but most important, phase of the reaction between influenza virus and its antiserum. This phase is interpreted as

the rapid development of an equilibrium in which a certain proportion of the virus particles is rendered non-infective by reversible union with antibody. It is followed by a slower and more variable inactivation, which is not reversible by simple dilution. There is insufficient information available to provide any interpretation of the nature of this phase.

No attempt will be made here to apply the results obtained with influenza virus to the general problem of the nature of the virus-antibody reaction, or discuss them in the light of previous work on other viruses. It is felt that the quantitative chorioallantoic technique is particularly suited to the study of problems of this sort, and with the accumulation of similar data for the other viruses which can be studied by this technique, it may be hoped that the results will eventually provide a useful contribution to the general problem.

SUMMARY.

The details of the interaction between egg passage influenza virus and ferret immune sera have been studied by the method of chorioallantoic membrane inoculation.

The reaction may be divided into two phases, the first and most important of which depends upon the rapid establishment of an equilibrium between virus, antibody, and inactivated virus, which conforms to the laws of mass action applicable to reversible unions in general.

There is a slower and more variable second phase of irreversible inactivation.

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